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<b>(54) Title:</b> EXPRESSED CHEMOKINES, THEIR PRODUCTION AND USES <b>(57) Abstract</b> <p>The present invention provides nucleotide and amino acid sequences that identify and encode novel expressed chemokines (ECs) from liver and pituitary gland tissues. The present invention also provides for antisense molecules to the nucleotide sequences which encode ECs, expression vectors for the production of purified ECs, antibodies capable of binding specifically to ECs, hybridization probes or oligonucleotides for the detection of EC-encoding nucleotide sequences, genetically engineered host cells for the expression of ECs, diagnostic tests for inflammation or disease based on EC-encoding nucleic acid molecules or antibodies capable of binding specifically to ECs, pharmaceutical compositions to treat inflammation or disease based on EC-encoding nucleic acid molecules or antibodies capable of binding specifically to ECs.</p>		

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## EXPRESSED CHEMOKINES, THEIR PRODUCTION AND USES

### CROSS-REFERENCE TO RELATED APPLICATIONS

- 5           This application is related to co-pending United States Applications Serial Nos. 08/303,241 filed September 7, 1994 and 08/320,011, filed October 5, 1994.

### BACKGROUND ART

- 10           Leukocytes including monocytes, macrophages, basophils, and eosinophils play important roles in the pathological mechanisms initiated by T and/or B lymphocytes. Macrophages, in particular, produce powerful oxidants and proteases which contribute to tissue destruction and secrete a range of cytokines which recruit and activate other inflammatory cells.

- The investigation of the critical, regulatory processes by which white cells proceed to their appropriate destination and interact with other cells is underway. The current model of leukocyte movement or trafficking from the blood to injured or inflamed tissues comprises the following steps. 15           The first step is the rolling adhesion of the leukocyte along the endothelial cells of the blood vessel wall. This movement is mediated by transient interactions between selectins and their ligands. A second step involves cell activation which promotes a more stable leukocyte-endothelial cell interaction mediated by the integrins and their ligands. This stronger, more stable adhesion precipitates the final steps of leukocyte diapedesis and extravasation into the tissues. 20

- The chemokine family of polypeptide cytokines, also known as intercrines, possesses the cellular specificity required to explain leukocyte trafficking in different inflammatory situations. First, chemokines mediate the expression of particular adhesion molecules on endothelial cells; and second, they generate gradients of chemoattractant factors which activate specific cell types. In 25           addition, the chemokines stimulate the proliferation of specific cell types and regulate the activation of cells which bear specific receptors. Both of these activities demonstrate a high degree of target cell specificity.

- The chemokines are small polypeptides, generally about 70-100 amino acids (aa) in length, 8-11 kD in molecular weight and active over a 1-100 ng/ml concentration range. Initially, they were 30           isolated and purified from inflamed tissues and characterized relative to their bioactivity. More recently, chemokines have been discovered through molecular cloning techniques and characterized by structural as well as functional analysis.

- The chemokines are related through a four cysteine motif which is based primarily on the spacing of the first two cysteine residues in the mature molecule. Currently the chemokines are 35           assigned to one of two families, the C-X-C chemokines ( $\alpha$ ) and the C-C chemokines ( $\beta$ ). Although exceptions exist, the C-X-C chemokines generally activate neutrophils and fibroblasts while the C-C chemokines act on a more diverse group of target cells which include monocytes/macrophages, basophils, eosinophils, T lymphocytes and others. The known chemokines of both families are synthesized by many diverse cell types and are reviewed in Thomson A. (1994) The Cytokine

Handbook, 2d Ed. Academic Press, NY. The two groups of chemokines will be described in turn.

At this time, the C-C chemokines number fewer than the C-X-C chemokines, and they appear to have less N-terminal processing. A brief description of human and murine C-C chemokines follows.

The macrophage inflammatory proteins alpha and beta (MIP-1 $\alpha$  and  $\beta$ ) were first purified from stimulated mouse macrophage cell line and elicited an inflammatory response when injected into normal tissues. At least three distinct and non-allelic genes encode human MIP-1 $\alpha$  and seven such genes encode MIP-1 $\beta$ .

MIP-1 $\alpha$  and MIP-1 $\beta$  consist of 68-69 aa which are about 70% identical in their acidic, mature secreted forms. They are both expressed in stimulated T cells, B cells and monocytes in response to mitogens, anti-CD3 and endotoxin, and both polypeptides bind heparin. While both molecules stimulate monocytes, MIP-1 $\alpha$  chemoattracts the CD-8 subset of T lymphocytes and eosinophils, while MIP-1 $\beta$  chemoattracts the CD-4 subset of T lymphocytes. In mouse, these proteins are known to stimulate myelopoiesis.

I-309 was cloned from a human  $\gamma$ - $\delta$  T cell line and shows 42% aa identity to T cell activation gene 3 (TCA3) cloned from mouse. There is considerable nucleotide homology between the 5' flanking regions of these two proteins, and they share an extra pair of cysteine residues not found in other chemokines. Such similarities suggest I-309 and TCA3 are species homologs which have diverged in sequence and function.

RANTES is another C-C chemokine which is expressed in T cells (but not B cells), in platelets, in some tumor cell lines, and in stimulated rheumatoid synovial fibroblasts. In the latter, it is regulated by interleukins-1 and -4, transforming nerve factor and interferon- $\gamma$ . The cDNA cloned from T cells encodes a basic 8 kD protein which lacks N-linked glycosylation and is able to affect lymphocytes, monocytes, basophils and eosinophils. The expression of RANTES mRNA is substantially reduced following T cell stimulation.

Monocyte chemotactic protein (MCP-1) is a 76 aa protein which appears to be expressed in almost all cells and tissues upon stimulation by a variety of agents. The targets of MCP-1, however, are limited to monocytes and basophils in which it induces an MCP-1 receptor, G protein-linked calcium flux (Charo I, personal communication). Two other related proteins, MCP-2 and MCP-3, were purified from a human osteosarcoma cell line. MCP-2 and MCP-3 have 62% and 73% aa identity, respectively, with MCP-1 and share its chemoattractant specificity for monocytes.

Current techniques for diagnosis of abnormalities in the inflamed or diseased tissues mainly rely on observation of clinical symptoms or serological analyses of body tissues or fluids for hormones, polypeptides or various metabolites. Mammals subject to conditions or diseases associated with inflammation often manifest no clinical symptoms at early stages of disease or tumor development. Furthermore, serological analyses do not always differentiate between invasive diseases and genetic syndromes which have overlapping or very similar ranges. Thus, development of new diagnostic techniques comprising the chemokines of the present invention would provide for early and accurate diagnoses, would give a better understanding of molecular pathogenesis, and could be used in the development of effective therapies.

The chemokine molecules are reviewed in Schall TJ (1994) Chemotactic Cytokines: Targets for Therapeutic Development. International Business Communications, Southborough MA pp 180-270; and in Paul WE (1993) Fundamental Immunology, 3rd Ed. Raven Press, NY pp 822-826.

## 5 **DISCLOSURE OF INVENTION**

The subject invention provides a nucleotide sequence which uniquely encodes a novel human protein from normal liver tissue. The new gene, which is known as liver expressed chemokine 1, or lvec-1 (Incyte Clone No. 87825), encodes a polypeptide designated LVEC-1, of the C-C chemokine family. The invention also comprises diagnostic tests for inflammatory conditions which include steps  
10 for testing a sample or an extract thereof with lvec-1 DNA, oligomers or fragments thereof. Aspects of the invention include lvec-1 antisense molecules; cloning or expression vectors containing nucleic acid encoding LVEC-1; host cells or organisms transformed with expression vectors containing nucleic acid encoding LVEC-1; purified LVEC-1; and methods for the production and recovery of purified LVEC-1 from host cells.

The subject invention provides a nucleotide sequence which uniquely encodes a novel human protein from normal liver tissue and an immortalized T and B cell hybrid. The new gene, which is known as liver expressed chemokine 2, or lvec-2 (Incyte Clone No. 88564), encodes a polypeptide designated LVEC-2, of the C-C chemokine family. The invention also comprises diagnostic tests for inflammatory conditions which include steps for testing a sample or an extract thereof with lvec-2  
20 DNA, oligomers or fragments thereof. Aspects of the invention include lvec-2 antisense molecules; cloning or expression vectors containing nucleic acid encoding LVEC-2; host cells or organisms transformed with expression vectors containing lvec-2; purified LVEC-2; and methods for the production and recovery of purified LVEC-2 from host cells.

The subject invention provides a nucleotide sequence which uniquely encodes a novel human protein from normal pituitary gland and liver. The new gene, which is known as pituitary gland expressed chemokine, or pgec (Incyte Clone No. 111571), encodes a polypeptide designated PGEC, of the C-C chemokine family. The invention also comprises diagnostic tests for inflammatory conditions which include steps for testing a sample or an extract thereof with pgec DNA, oligomers or fragments thereof. Aspects of the invention include pgec antisense molecules; cloning or expression vectors  
30 containing nucleic acid encoding PGEC; host cells or organisms transformed with expression vectors containing nucleic acid encoding PGEC; purified PGEC; and methods for the production and recovery of purified PGEC from host cells.

## **BRIEF DESCRIPTION OF THE DRAWINGS**

Figure 1 displays the nucleotide sequence for liver expressed chemokine 1 (lvec-1) and the predicted amino acid sequence of LVEC-1 (SEQ ID NO:1 and SEQ ID NO:2, respectively).

Figure 2 provides an analysis of biochemical characteristics of LVEC-1 based on the predicted amino acid sequence and composition.

Figure 3 displays an analysis of hydrophobicity and immunogenic characteristics of LVEC-1

based on the predicted aa sequence and composition.

Figure 4 displays the nucleotide sequence of liver expressed chemokine 2 (lvec-2) and the predicted amino acid sequence of LVEC-2 (SEQ ID NO:3 and SEQ ID NO:4, respectively).

5 Figure 5 provides an analysis of biochemical characteristics of LVEC-2 based on the predicted amino acid sequence and composition.

Figure 6 displays an analysis of hydrophobicity and immunogenic characteristics of LVEC-2 based on the predicted amino acid sequence and composition.

Figure 7 displays the nucleotide sequence of pituitary expressed chemokine (pgec) and the predicted amino acid sequence of PGEC (SEQ ID NO:5 and SEQ ID NO:6, respectively).

10 Figure 8 provides an analysis of biochemical characteristics of PGEC based on the predicted amino acid sequence and composition.

Figure 9 displays an analysis of hydrophobicity and immunogenic characteristics of PGEC based on the predicted amino acid sequence and composition.

15 Figure 10 shows the aa alignment of LVEC-1, LVEC-2, and PGEC with other human chemokines of the C-C family. Alignments shown were produced using the multisequence alignment program of DNASTAR software.

Figure 11 illustrates the change in intracellular calcium in response to PGEC (clone number 111571) in THP-1 cells.

20 Figure 12 illustrates the chemotaxis of THP-1 cells in response to PGEC (clone number 111571).

Figure 13 illustrates the desensitization of PGEC (clone number 111571) and MIP-1 alpha in THP-1 cells.

## 25 **MODES FOR CARRYING OUT THE INVENTION**

### **Definitions**

As used herein, the term "expressed chemokine" (EC) refers to a polypeptide of the present invention, i.e., LVEC-1, LVEC-2 or PGEC, or active fragments thereof, which are encoded by an mRNA transcribed from the nucleic acid of SEQ ID NO:1, SEQ ID NO:3 and SEQ ID NO:5, respectively. The EC  
30 may be naturally occurring or chemically synthesized.

As used herein, the term "active" refers to those forms of an EC which retain the biologic and/or immunologic activities of the naturally occurring EC.

As used herein, the term "naturally occurring EC" refers to an EC produced by human cells that have not been genetically engineered and specifically contemplates various EC forms arising from  
35 post-translational modifications of the polypeptide including but not limited to acetylation, carboxylation, glycosylation, phosphorylation, lipidation and acylation.

As used herein, the term "derivative" refers to a polypeptide derived from naturally occurring EC by a chemical modification such as ubiquitination, labeling (e.g., with radionuclides, various enzymatic modifications), pegylation (derivatization with polyethylene glycol) or by insertion

or substitution by chemical synthesis of amino acids, such as, for example, ornithine, which do not normally occur in human proteins.

As used herein, the term "variant" or "recombinant variant" refers to any polypeptide differing from naturally occurring EC by amino acid insertions, deletions, and/or substitutions, created using recombinant DNA techniques. Guidance in determining which amino acid residues may be replaced, added or deleted without abolishing activities of interest, such as, for example, cell adhesion and/or chemotaxis, may be found by comparing the sequence of the EC of interest with that of homologous cytokines and minimizing the number of amino acid sequence changes made in regions of high homology.

Preferably, amino acid substitutions are the result of replacing one amino acid with another amino acid having similar structural and/or chemical properties, such as the replacement of a leucine with an isoleucine or valine, an aspartate with a glutamate, or a threonine with a serine, i.e., conservative amino acid replacements. Insertions or deletions are typically in the range of about 1 to 5 amino acids. The variation allowed may be experimentally determined by systematically making insertions, deletions, or substitutions of amino acids in an EC of interest using recombinant DNA techniques known to those of skill in the art and assaying the resulting recombinant variants for activity.

Where desired, an EC of the present invention can be genetically engineered to contain a "signal or leader sequence" that can direct the polypeptide through the membrane of a cell. As will be understood by those of skill in the art, such a sequence may be naturally occurring on the polypeptides of the present invention or provided from heterologous protein sources by recombinant DNA techniques.

As used herein, an EC "fragment", "portion" or "segment" refers to any stretch of amino acids which has sufficient length to display biologic and/or immunologic activity and in preferred embodiments will contain at least about 5 amino acids, at least about 7 amino acids, at least about 8 to 13 amino acids and in additional embodiments about 17 or more amino acids.

As used herein, an "oligonucleotide" or polynucleotide "fragment", "portion," or "segment" refers to any stretch of nucleic acids encoding the ECs of the present invention which is of sufficient length to use as a primer in polymerase chain reaction (PCR), or various hybridization procedures known to those of skill in the art, for the purpose of identifying or amplifying identical or related nucleic acids.

The present invention includes vectors and host cells transformed with recombinant nucleic acid molecules encoding the ECs of the present invention and purified EC polypeptides from natural or recombinant sources. Various methods for the isolation of the EC polypeptides of the present invention are known by those of skill in the art. For example, EC polypeptides may be purified by immunoaffinity chromatography by employing the antibodies provided by the present invention. Various other methods of protein purification well known in the art include those described in Deutscher M (1990) Methods in Enzymology Vol. 182, Academic Press, San Diego; and Scopes R (1982) Protein Purification: Principles and Practice. Springer-Verlag, New York, both incorporated herein by reference.

As used herein the term "recombinant" refers to a polynucleotide of the present invention

which encodes an EC and which is prepared using recombinant DNA techniques. The nucleic acid which encodes an EC may also include allelic or recombinant variants and mutants thereof.

As used herein, the term "probe" or "nucleic acid probe" or "polynucleotide probe" or "oligonucleotide probe" refers to a portion, fragment, or segment of lvec-1, lvec-2 or pgec that is capable of being hybridized to a desired target sequence. A probe can be used to detect, amplify, or quantify cDNAs or endogenous nucleic acid encoding ECs of the present invention, i.e., LVEC-1, LVEC-2 or PGEC, by employing conventional techniques in molecular biology. A probe may be of variable length, preferably from about 10 nucleotides up to several hundred nucleotides. As will be understood by those of skill in the art, hybridization conditions and probe design will vary depending upon the intended use. For example, a probe intended for use in PCR will be from about 15 to 30 nucleotides in length and may be part of a pool of degenerate probes, i.e., oligonucleotides which tolerate nucleotide mismatch but accommodate binding to an unknown sequence; whereas a probe for use in Southern or northern hybridizations may be a single, specific nucleotide sequence that is several hundred nucleotides in length. Accordingly, a preferred probe for the specific detection of lvec-1, lvec-2 or pgec, comprises a polynucleotide or oligonucleotide fragment from a non-conserved nucleotide region of SEQ ID NO: 1, SEQ ID NO: 3 or SEQ ID NO: 5, respectively. As used herein, the term "non-conserved nucleotide region" refers to a nucleotide region that is unique to SEQ ID NO: 1, SEQ ID NO:3 or SEQ ID NO:5 and does not comprise a region that is conserved in the family of C-C chemokines. Probes may be single-stranded or double-stranded and may have specificity in solution, cell, tissue or membrane-based hybridizations including in situ and ELISA-like technologies. The present invention encompasses oligonucleotides, fragments or portions of the polynucleotides disclosed herein, or their complementary strands used as probes.

"Oligonucleotides" or "oligonucleotide probes" are prepared based on the nucleotide sequences disclosed herein which encode ECs of the present invention. Oligonucleotides comprise portions of the nucleotide sequences disclosed herein and contain at least about 15 nucleotides, and usually at least about 20 nucleotides and may include up to 60 nucleotides. Nucleic acid probes may comprise portions of the sequence having fewer nucleotides than about 6 kb, usually fewer than about 1 kb. The oligonucleotides and nucleic acid probes of the present invention may be used to determine whether nucleic acid encoding a particular EC is present in a cell or tissue or to isolate similar nucleic acid sequences from chromosomal DNA as described by Walsh PS et al (1992) PCR Methods Appl 1:241-250.

Nucleic acid probes of the present invention may be derived from naturally occurring nucleic acids, recombinant single- or double-stranded nucleic acids, or may be chemically synthesized. Probes may be labeled by nick translation, Klenow fill-in reaction, PCR or other methods well known in the art. Preparation and labeling of the nucleic acid probes of the present invention are described in Sambrook J. et al. (1989) Molecular Cloning: A Laboratory Manual, 2d Ed., Cold Spring Harbor, NY; and Ausubel FM et al (1989) Current Protocols in Molecular Biology, Vol 2. John Wiley & Sons, both incorporated herein by reference.

Alternatively, recombinant variants encoding the polypeptides of the present invention or



related polypeptides may be synthesized or identified through hybridization techniques known by those of skill in the art by making use of the "redundancy" in the genetic code. Various codon substitutions, such as the silent changes which produce various restriction sites, may be introduced to optimize cloning into a plasmid or viral vector or for expression in a particular prokaryotic or eukaryotic system. Mutations in a polypeptide of the present invention may also be introduced to modify the properties of the polypeptide, such as, for example, to change receptor-binding affinities, or polypeptide degradation or turnover rate.

#### Detailed Description of the Invention

The present invention provides nucleotide sequences disclosed herein which uniquely identify novel chemokines of the C-C family, LVEC-1, LVEC-2, and PGEC. LVEC-1 has been identified in a cDNA library made from liver tissue. LVEC-2 has been identified in a cDNA library made from liver tissue; in cDNA libraries made from macrophages untreated and treated with lipopolysaccharide (LPS), THP-1 monocytes treated with phorbol myristic acid (PMA) and LPS; and T and B lymphoblasts from a leukemia source; and in a cDNA library made from fetal lung tissue. PGEC has been identified in a cDNA library made from pituitary gland tissue; a cDNA library made from liver tissue; a cDNA library made from uterus tissue; a cDNA library made from prostate tissue; a cDNA library made from spleen tissue; and a cDNA library from breast tissue.

When an EC is specifically expressed in the tissue from which it was identified and has not been found in other tissues, it is useful to have a diagnostic test for each particular EC. Excessive expression of the ECs of the present invention leads to attraction of neutrophils, monocytes/macrophages and/or T and B lymphocytes to the area and induces their production of excess proteases and other molecules which can lead to tissue damage or destruction. Therefore, a diagnostic test for excess expression of a particular EC can accelerate diagnosis and proper treatment of the inflammation before extensive tissue damage or destruction occurs.

Nucleotide sequences encoding ECs of the present invention, or their complements, have numerous applications in techniques known to those skilled in the art of molecular biology. These techniques include use as hybridization probes, use for chromosome and gene mapping, use in the recombinant production of ECs, and use in generation of anti-sense RNA and DNA or their chemical analogs and the like. Uses of oligonucleotides encoding ECs disclosed herein are exemplary of known techniques and are not intended to limit their use in any technique known to a person of ordinary skill in the art. Furthermore, the nucleotide sequences disclosed herein may be used in molecular biology techniques that have not yet been developed, provided the new techniques rely on properties of polynucleotide sequences that are currently known, e.g., for example, the triplet genetic code and specific base pair interactions.

It will be appreciated by those skilled in the art that as a result of the degeneracy of the genetic code, a multitude of EC-encoding nucleotide sequences, some bearing minimal nucleotide sequence homology to the nucleotide sequence of any known and naturally occurring EC gene, may be produced as long as the nucleotide sequence encodes an EC of the present invention. The invention has

specifically contemplated each and every possible variation of nucleotide sequence that could be made by selecting combinations based on possible codon choices. These combinations are made in accordance with the standard triplet genetic code as applied to the nucleotide sequence of naturally occurring EC, and all such variations are to be considered as being specifically disclosed.

5           Although nucleotide sequences which encode ECs and/or EC variants are preferably capable of hybridizing to the nucleotide sequence of the naturally occurring EC gene under stringent conditions, it may be advantageous to produce nucleotide sequences encoding EC or EC derivatives possessing a substantially different codon usage. Codons can be selected to increase the rate at which expression of the peptide occurs in a particular prokaryotic or eukaryotic expression host in accordance with the  
10 frequency with which a particular codon is utilized by the host. Other reasons for substantially altering the nucleotide sequence encoding ECs and/or EC derivatives of the present invention without altering their encoded amino acid sequence include the production of RNA transcripts having more desirable properties, e.g., a greater half-life, than transcripts produced from the naturally occurring nucleotide sequence.

15           Nucleotide sequences encoding ECs of the present invention may be joined to a variety of other nucleotide sequences by means of well established recombinant DNA techniques (Sambrook J et al. (1989) Molecular Cloning: A Laboratory Manual, 2d Ed. Cold Spring Harbor, NY).

          Useful nucleotide sequences for joining to EC sequences include an assortment of cloning vectors, e.g., plasmids, cosmids, lambda phage derivatives, phagemids, and the like, that are known in  
20 the art. Vectors of interest include expression vectors, replication vectors, probe generation vectors, sequencing vectors, and the like. In general, vectors of interest may contain an origin of replication functional in at least one organism, convenient restriction endonuclease sensitive sites, and selectable markers for the host cell.

          Another aspect of the subject invention is to provide for EC-specific nucleic acid hybridization  
25 probes capable of hybridizing with naturally occurring nucleotide sequences encoding ECs. Such probes for the detection of similar EC encoding sequences should preferably contain at least 50% of the nucleotides from a C-X-C or C-C encoding sequence. Such probes for the detection of EC encoding sequences should preferably contain a nucleotide fragment from a non-conserved region of SEQ ID NO:1, SEQ ID NO: 3 or SEQ ID NO: 5. The hybridization probes of the subject invention may be derived  
30 from the nucleotide sequences of the SEQ ID NOs 1, 3, and 5, or from genomic sequences including promoters, enhancer elements and introns of naturally occurring ECs. Hybridization probes may be labeled by a variety of reporter groups, including radionuclides such as <sup>32</sup>P or <sup>35</sup>S, or enzymatic labels such as alkaline phosphatase, coupled to the probe via avidin/biotin coupling systems, and the like through techniques known to those of skill in the art.

35           PCR as described in U.S. Patents 4,965,188 and 4,683,195 and 4,800,195 provides additional uses for oligonucleotides based upon the nucleotide sequences disclosed herein which encode ECs. Such probes used in PCR may be of recombinant origin, may be chemically synthesized, or a mixture of both, and will comprise a discrete nucleotide sequence for diagnostic use for the identification of an EC of the present invention or a degenerate pool of possible sequences for identification of closely related

genomic sequences.

Other means of producing EC-specific hybridization probes include the cloning of nucleic acid sequences encoding ECs and EC derivatives into vectors for the production of mRNA probes. Such vectors are known in the art and are commercially available and may be used to synthesize RNA probes in vitro by means of the addition of the appropriate RNA polymerase, such as T7 or SP6 RNA polymerase, and the appropriate radioactively labeled nucleotides.

It is now possible to produce a DNA sequence, or portions thereof, encoding EC and EC derivatives entirely by synthetic chemistry, after which the gene can be inserted into any of the many available DNA vectors using reagents, vectors and cells that are known in the art. Synthetic chemistry may be used to reproduce the entire sequence of an EC encoding gene, any portion thereof, or to introduce mutations into the sequence.

Methods for DNA sequencing are well known in the art. Conventional enzymatic methods employ DNA polymerase Klenow fragment, SEQUENASE™ or Taq polymerase to extend DNA chains from an oligonucleotide primer annealed to the DNA template of interest. Methods have been developed for the use of both single- and double- stranded templates. The chain termination reaction products are usually electrophoresed on urea-acrylamide gels and are detected either by autoradiography (for radionuclide-labeled precursors) or by fluorescence (for fluorescent-labeled precursors). Recent improvements in mechanized reaction preparation, sequencing and analysis using the fluorescent detection method have permitted expansion in the number of sequences that can be determined per day (using machines such as the Catalyst 800 and the Applied Biosystems 373 DNA sequencer).

The nucleotide sequence for a particular EC can be used to construct an assay to detect inflammation and disease associated with abnormal levels of expression of that EC. The nucleotide sequence can be labeled by methods known in the art and added to a fluid or tissue sample from a patient under hybridizing conditions. After an incubation period, the sample is washed with compatible fluid which optionally contains a dye if the nucleotide has been labeled with an enzyme or other label or reporter molecule requiring a developer. If the nucleotide sequence hybridizes with the sample, the dye is detected. The amount of dye detected is compared with a standard. If the amount of dye varies significantly from the standard, EC is present at an abnormal level and may indicate the presence of inflammation or disease.

The nucleotide sequence encoding an EC can be used to construct hybridization probes for mapping the gene which encodes that EC and for the genetic analysis of individuals with EC genetic disorders, allelic variants or other genetic traits of interest. The nucleotide sequences provided herein may be mapped to a chromosome and specific regions of a chromosomes using well known genetic and/or chromosomal mapping techniques. These techniques include in situ hybridization, linkage analysis against known chromosomal markers, hybridization screening with libraries or flow-sorted chromosomal preparations specific to known chromosomes, and the like. The technique of fluorescent in situ hybridization of chromosome spreads has been described, among other places, in Verma et al (1988) Human Chromosomes: A Manual of Basic Techniques, Pergamon Press, NY. Fluorescent in situ hybridization of chromosomal preparations and other physical chromosome mapping techniques may be

correlated with additional genetic map data. Examples of genetic map data can be found in O'Brien (1990) Genetic Maps: Locus Maps of Complex Genomes, Book 5: Human Maps, Cold Spring Harbor Laboratory, NY. Correlation between the location of a gene encoding an EC on a physical chromosomal map and a specific disease (or predisposition to a specific disease) can help detect genetic diseases and carrier states. The nucleotide sequence of the subject invention can be used to detect differences in gene sequence between normal individuals and individuals subject to a disease or condition.

Nucleotide sequences encoding ECs may be used to produce purified ECs using well known methods of recombinant DNA technology. Among the many publications that teach methods for the expression of genes after they have been isolated is Goeddel (1990) Gene Expression Technology, Methods and Enzymology, Vol 185, Academic Press, San Diego. ECs may be expressed in a variety of host cells, including cells of prokaryotic or eukaryotic origin. Host cells may be from species either the same or different than the species from which the nucleotide sequences encoding EC are endogenous. Advantages of producing the EC by recombinant DNA technology include obtaining highly enriched sources of the proteins for purification and the availability of simplified purification procedures. An EC of the present invention may be expressed as a chimeric protein with one or more additional polypeptide domains added to facilitate protein purification. Such purification facilitating domains include, but are not limited to, metal chelating peptides such as histidine-tryptophan modules that allow purification on immobilized metals, protein A domains that allow purification on immobilized immunoglobulin, and the domain utilized in the FLAGS extension/affinity purification system (Immunex Corp., Seattle WA). The inclusion of a cleavable linker sequence (such as Factor XA or enterokinase) between the purification domain and the EC-encoding sequence may be useful to facilitate production of EC.

Cells transformed with DNA encoding EC may be cultured under conditions suitable for the expression of the EC and the recovery of the protein from the cell culture. EC produced by a recombinant cell may be secreted or may be contained intracellularly, depending on the particular genetic construction used. In general, it is more convenient to prepare recombinant proteins in secreted form. Purification steps will depend on the nature of the production process used and the particular EC produced.

Translation of any of the cloned chemokine cDNAs of the present invention, i.e., lvec-1, lvec-2 or pgec, into protein may be accomplished by subcloning the cDNA into an appropriate expression vector and transfecting this vector into an appropriate expression host. As described in Example VII, a preferred expression vector for the expression and purification of LVEC-1, LVEC-2 and PGEC is one which provides for expression of a fusion protein comprising a chemokine of the present invention and contains nucleic acid encoding 6 histidine residues followed by thioredoxin and an enterokinase cleavage site. The histidine residues facilitate purification on IMIAC (immobilized metal ion affinity chromatography as described in Porath et al. (1992) Protein Expression and Purification 3:263-281) while the enterokinase cleavage site provides a means for purifying the chemokine from the fusion protein.

The expression vector used for the generation of the cDNA libraries described herein, which

provides a promoter for  $\beta$ -galactosidase upstream of the cloning site, followed by a nucleotide sequence containing the amino-terminal Met and the subsequent 7 residues of  $\beta$ -galactosidase followed by a bacteriophage promoter useful for artificial priming and transcription and a number of unique restriction sites (including EcoRI), can also be used for expression of the chemokines of the present invention. Induction of the isolated bacterial strain with IPTG using standard methods will produce a fusion protein corresponding to the first seven residues of  $\beta$ -galactosidase, about 15 residues of linker, and the EC encoded within the cDNA. Since cDNA clone inserts are generated by an essentially random process, there is one chance in three that the included cDNA will lie in the correct frame for proper translation. If the cDNA is not in the proper reading frame, it can be obtained by deletion or insertion of the appropriate number of bases by well known methods including in vitro mutagenesis, digestion with exonuclease III or mung bean nuclease, or oligonucleotide linker inclusion. The EC molecule of interest will be expressed in the bacterial system as described.

An EC encoding nucleotide sequence of the present invention can be shuttled to vectors known to be useful for expression of protein in specific hosts. Oligonucleotide amplimers containing cloning sites as well as a segment of DNA sufficient to hybridize to stretches at both ends of the target cDNA (25 bases) can be synthesized chemically by standard methods. These primers can then be used to amplify the desired gene segments by PCR. The resulting new gene segments can be digested with appropriate restriction enzymes under standard conditions and isolated by gel electrophoresis. Alternately, similar gene segments can be produced by digestion of the nucleotide sequence with appropriate restriction enzymes and filling in the missing gene segments with chemically synthesized oligonucleotides. Segments of the coding sequence from more than one gene can be ligated together and cloned in appropriate vectors to optimize expression of recombinant sequence.

Suitable expression hosts for such chimeric molecules include but are not limited to mammalian cells such as Chinese Hamster Ovary and human 293 cells, insect cells such as Sf9 cells, yeast cells such as Saccharomyces cerevisiae, and bacteria such as E. coli. For each of these cell systems, a useful expression vector may also include an origin of replication to allow propagation in bacteria and a selectable marker, such as the  $\beta$ -lactamase antibiotic resistance gene, to allow selection in bacteria. In addition, the vectors may include a second selectable marker, such as the neomycin phosphotransferase gene, to allow selection in transfected eukaryotic host cells. Vectors for use in eukaryotic expression hosts may require RNA processing elements such as 3' polyadenylation sequences if such are not part of the cDNA of interest.

Additionally, the vector may contain promoters or enhancers which increase gene expression. Such promoters are host specific and include MMTV, SV40, or metallothionine promoters for CHO cells; trp, lac, tac or T7 promoters for bacterial hosts, or alpha factor, alcohol oxidase or PGH promoters for yeast. Transcription enhancers, such as the RSV enhancer, may be used in mammalian host cells. Once homogeneous cultures of recombinant cells are obtained through standard culture methods, large quantities of recombinantly produced ECs can be recovered from the conditioned medium and analyzed using chromatographic methods known in the art.

In addition to recombinant production, EC fragments may be produced by direct peptide

synthesis using solid-phase techniques (cf Stewart et al (1969) Solid-Phase Peptide Synthesis, WH Freeman Co. San Francisco; Merrifield R (1963) J Am Chem Soc 85:2149-2154. In vitro protein synthesis may be performed using manual techniques or by automation. Automated synthesis may be achieved, for example, using Applied Biosystems 431A Peptide Synthesizer (Foster City, California) in accordance with the instructions provided by the manufacturer. Various fragments of ECs may be chemically synthesized separately and combined using chemical methods to produce full length ECs .

ECs for use in the induction of antibodies must have immunogenic activity. Peptides for use in the induction of EC-specific antibodies will comprise an amino acid sequence consisting of at least five amino acids and preferably at least 10 amino acids such that the peptide retains the three-dimensional configuration of a portion of the naturally occurring EC and may contain the entire amino acid sequence of the naturally occurring EC. Short stretches of EC aa may be fused with those of another protein such as keyhole limpet hemocyanin and the chimeric molecule used for antibody production.

Various methods are known to those of skill in the art for preparing monoclonal and polyclonal antibodies to ECs of the present invention. In one approach, denatured EC from the reverse phase HPLC separation is obtained and used to immunize mice or rabbits using techniques known to those of skill in the art. About 100 micrograms are adequate for immunization of a mouse, while up to 1 mg can be used for immunization of a rabbit. For identifying mouse hybridomas, the denatured protein can be radiiodinated and used to screen potential murine B-cell hybridomas for those which produce antibody. This procedure requires only small quantities of protein, such that 20 mg would be sufficient for labeling and screening of several thousand clones.

In another approach, the amino acid sequence of EC, as deduced from the cDNA sequence, is analyzed to determine regions of high immunogenicity. Polypeptides comprising these regions are synthesized and used in suitable immunization protocols to raise antibodies. Analysis to select appropriate epitopes is described by Ausubel FM et al (1989, Current Protocols in Molecular Biology, Vol 2. John Wiley & Sons). The optimal amino acid sequences for immunization are usually at the C-terminus, the N-terminus and those intervening, hydrophilic regions of the polypeptide which are likely to be exposed to the external environment when the protein is in its natural conformation.

Typically, selected peptides, about 15 residues in length, are synthesized using an Applied Biosystems Peptide Synthesizer Model 431A using fmoc-chemistry and coupled to keyhole limpet hemocyanin (KLH, Sigma) by reaction with M- maleimidobenzoyl-N-hydroxysuccinimide ester (MBS; cf. Ausubel FM et al, supra). If necessary, a cysteine may be introduced at the N-terminus of the peptide to permit coupling to KLH and animals are immunized with the peptide-KLH complex in complete Freund's adjuvant. The resulting antisera are tested for antipeptide activity by binding the peptide to plastic, blocking with 1% BSA, reacting with antisera, washing and reacting with labeled (radioactive or fluorescent), affinity purified, specific goat anti-rabbit IgG.

Hybridomas may also be prepared and screened using standard techniques. Hybridomas of interest are detected by screening with labeled EC to identify those fusions producing the monoclonal antibody with the desired specificity. For example, in a typical protocol, wells of plates (FAST, Becton-Dickinson, Palo Alto, CA) are coated with affinity purified, specific rabbit-anti-mouse (or

suitable anti-species Ig) antibodies at about 10 mg/ml. The coated wells are blocked with 1% BSA, washed and exposed to supernatants from hybridomas. After incubation the wells are exposed to labeled EC at a concentration of about 1 mg/ml. Clones producing antibodies will bind a quantity of labeled EC which is detectable above background. Such clones are expanded and subjected to 2 cycles of cloning at limiting dilution (1 cell/3 wells). Cloned hybridomas are injected into pristane-treated mice to produce ascites, and monoclonal antibody is purified from mouse ascitic fluid by affinity chromatography using Protein A. Monoclonal antibodies with affinities of at least  $10^8 \text{ M}^{-1}$ , preferably  $10^9$  to  $10^{10}$  or stronger, will typically be made by standard procedures as described in Harlow and Lane (1988) *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory, NY, or Goding (1986) *Monoclonal Antibodies: Principles and Practice*, 2d Ed. Academic Press New York City, both incorporated herein by reference.

Antibodies specific for a particular EC may be produced by inoculation of an appropriate animal with the EC. An antibody is specific for an EC if the antibody is produced against all or part of the EC polypeptide and binds to all or part of the protein. Induction of antibodies includes not only the stimulation of an immune response by injection into animals, but also analogous steps in the production of synthetic antibodies or other specific-binding molecules such as the screening of recombinant immunoglobulin libraries (cf. Orlandi et al (1989) *PNAS* 86:3833-3837, or Huse et al (1989) *Science* 256:1275-1281) or the *in vitro* stimulation of lymphocyte populations. Current technology (Winter and Milstein (1991) *Nature* 349:293-299) provides for a number of highly specific binding reagents based on the principles of antibody formation. These techniques may readily be adapted to produce molecules capable of specifically binding ECs.

Antibodies, inhibitors, antisense molecules, receptors or analogs of the various ECs (treatments for excessive EC production, hereafter abbreviated "TEC") can provide different effects when administered therapeutically. The TECs will be formulated in a nontoxic, inert, pharmaceutically acceptable aqueous carrier medium preferably at a pH of about 5 to 8, more preferably 6 to 8, although the pH may vary according to the characteristics of the antibody, inhibitor, receptor or analog being formulated and the condition to be treated. Characteristics of the TEC include solubility of the molecule, half-life and antigenicity/immunogenicity and may aid in defining an effective carrier. Naturally occurring human proteins are preferred as TECs, but organic molecules resulting from drug screens may be equally effective in particular situations.

TECs may be delivered by known routes of administration including but not limited to topical creams or gels; transmucosal spray or aerosol, transdermal patch or bandage; injectable, intravenous or lavage formulations; or orally administered liquids or pills. The particular formulation, exact dosage, and route of administration will be determined by the attending physician and will vary according to each specific situation.

Such determinations are made by considering multiple variables such as the condition to be treated, the TEC to be administered, and the pharmacokinetic profile of the particular TEC. Additional factors which may be taken into account include the severity of the disease state, the patient, age, weight, gender, diet, time of administration, drug combination, reaction sensitivities, and

tolerance/response to therapy. Long acting TEC formulations might be administered every 3 to 4 days, every week, or once every two weeks depending on half-life and clearance rate of the particular TEC.

Normal dosage amounts may vary from 0.1 to 100,000 micrograms, up to a total dose of about 1 g, depending upon the route of administration. Guidance as to particular dosages for the TECs is provided in the literature; see U.S. Patents 4,657,760; 5,206,344; or 5,225,212. It is anticipated that different formulations will be effective for different TECs and that administration targeting the liver may necessitate delivery in a manner different from that for delivery targeted to the pituitary gland.

It is contemplated that a liver condition or disease which activates leukocytes, particularly monocytes and macrophages, and precipitates permanent damage may be treatable with TECs. These conditions or diseases may be specifically diagnosed by the diagnostic tests discussed *infra*, such testing should be performed in patients with fatty liver, jaundice, hepatitis, cirrhosis, amyloidosis, and cancer. Similarly treatable conditions or diseases of the pituitary can be diagnosed by specific testing, which should be performed for patients with adenoma or multiple endocrine neoplasia, as well as other genetic or invasive conditions that activate cells which may destroy or compromise the function of the pituitary gland.

The examples below are provided to illustrate the subject invention. These examples are provided by way of illustration and are not included for the purpose of limiting the invention.

## EXAMPLES

### I Isolation of mRNA and construction of cDNA libraries

The cDNA sequences which encode LVEC-1 and LVEC-2 were initially identified among the partial nucleotide sequences comprising the normal liver library. Poly A mRNA was isolated from the liver of a 49 year old, Caucasian male (Catalogue #937220; Stratagene, 11011 N. Torrey Pines Rd., La Jolla, CA 92037) and used to construct a custom cDNA library as described below.

The cDNA which encodes PGEC was initially identified among the partial sequences comprising the pituitary gland library. Poly A mRNA was isolated from a pooled sample of 21 whole, normal pituitary glands from human brains of Caucasian males and females with a range of ages from 16-70 years. The poly A+ mRNA was obtained from Clontech Laboratories Inc. (Catalogue #6584-1 and #6584-2, 4030 Fabian Way, Palo Alto, CA 94303) and used to construct a cDNA library as described below.

The liver and pituitary gland cDNA libraries were constructed by Stratagene (11011 N. Torrey Pines Rd., La Jolla, CA 92037) using poly A mRNA. cDNA synthesis was primed using oligo dT and/or random hexamers. Synthetic adapter oligonucleotides were ligated onto cDNA ends enabling its insertion into the UNI-ZAP<sup>TM</sup> vector system (Stratagene). This allows high efficiency unidirectional (sense orientation) lambda library construction and the convenience of a plasmid system with blue/white color selection to detect clones with cDNA insertions. The quality of the each cDNA library was screened using either DNA probes or antibody probes, and then the pBluescript<sup>®</sup> phagemid



(Stratagene) was rapidly excised in living cells. The phagemid allows the use of a plasmid system for easy insert characterization, sequencing, site-directed mutagenesis, the creation of unidirectional deletions and expression of fusion proteins. Phage particles from each library were infected into the *E. coli* host strain XL1-BLUE® (Stratagene). The high transformation efficiency of XL1-BLUE® increases the probability of obtaining rare, under-represented clones from the cDNA library.

## II Isolation of cDNA Clones

The phagemid forms of individual cDNA clones were obtained by the *in vivo* excision process, in which a host *E. coli* strain, (XL1-BLUE® MRF) was coinfectd with an f1 helper phage. Proteins derived from both lambda phage and f1 helper phage initiate new DNA synthesis from defined sequences on the lambda target DNA and create a smaller, single stranded circular phagemid DNA molecule that includes all DNA sequences of the pBluescript® plasmid and the cDNA insert. The phagemid DNA is released from the cells and purified, then used to re-infect fresh bacterial host cells (SOLR), where the double stranded phagemid DNA was produced. Because the phagemid carries the gene for  $\beta$ -lactamase, the newly transformed bacteria are selected on medium containing ampicillin.

Phagemid DNA was purified using the QIAWELL-8 Plasmid Purification System from QIAGEN® DNA Purification System (QIAGEN Inc., 9259 Eton Ave., Chatsworth, CA 91311). This technique provides a rapid and reliable high-throughput method for lysing the bacterial cells and isolating highly purified phagemid DNA. The DNA eluted from the purification resin is suitable for DNA sequencing and other analytical manipulations.

## III Sequencing of cDNA Clones

The cDNA inserts from random isolates of the liver and pituitary gland libraries were sequenced in part. The cDNAs were sequenced by the method of Sanger F and AR Coulson (1975; J. Mol. Biol. 94:4411), using a Hamilton Micro Lab 2200 (Hamilton, Reno NV) in combination with four Peltier Thermal Cyclers (PTC200 from MJ Research, Watertown MA) and Applied Biosystems 377 or 373 DNA Sequencing Systems (Perkin Elmer) and reading frame was determined.

## IV Homology Searching of cDNA Clones and Deduced Protein

Each cDNA was compared to sequences in GenBank using a search algorithm developed by Applied Biosystems and incorporated into the INHERIT™ 670 Sequence Analysis System. In this algorithm, Pattern Specification Language (TRW Inc, Los Angeles CA) was used to determine regions of homology. The three parameters that determine how the sequence comparisons run were window size, window offset, and error tolerance. Using a combination of these three parameters, the DNA database was searched for sequences containing regions of homology to the query sequence, and the appropriate sequences were scored with an initial value. Subsequently, these homologous regions were examined using dot matrix homology plots to distinguish regions of homology from chance matches. Smith-Waterman alignments were used to display the results of the homology search.

Peptide and protein sequence homologies were ascertained using the INHERIT™ 670 Sequence Analysis System in a way similar to that used in DNA sequence homologies. Pattern Specification

Language and parameter windows were used to search protein databases for sequences containing regions of homology which were scored with an initial value. Dot-matrix homology plots were examined to distinguish regions of significant homology from chance matches.

BLAST, which stands for Basic Local Alignment Search Tool (Altschul SF (1993) J Mol Evol 36:290-300; Altschul, SF et al (1990) J Mol Biol 215:403-10), was used to search for local sequence alignments. BLAST produces alignments of both nucleotide and amino acid sequences to determine sequence similarity. Because of the local nature of the alignments, BLAST is especially useful in determining exact matches or in identifying homologs. BLAST is useful for matches which do not contain gaps. The fundamental unit of BLAST algorithm output is the High-scoring Segment Pair (HSP).

An HSP consists of two sequence fragments of arbitrary but equal lengths whose alignment is locally maximal and for which the alignment score meets or exceeds a threshold or cutoff score set by the user. The BLAST approach is to look for HSPs between a query sequence and a database sequence, to evaluate the statistical significance of any matches found, and to report only those matches which satisfy the user-selected threshold of significance. The parameter E establishes the statistically significant threshold for reporting database sequence matches. E is interpreted as the upper bound of the expected frequency of chance occurrence of an HSP (or set of HSPs) within the context of the entire database search. Any database sequence whose match satisfies E is reported in the program output.

#### 20 V Identification and Full Length Sequencing of EC

From all of the randomly picked and sequenced clones of the liver library, two sequences were homologous to but clearly different from one another and from known C-C chemokine molecules. These sequences were found within Incyte clones 87825 and 88564 and have been designated lvec-1 and lvec-2, respectively. When all three possible predicted translations of the sequence were searched 25 against protein databases such as SwissProt and PIR, no exact matches were found to either of the expressed proteins, LVEC-1 and LVEC-2.

From all of the randomly picked and sequenced clones of the pituitary gland library, only one sequence was homologous to but clearly different from known C-C chemokine molecules. This sequence was found within Incyte clone 111571 and has been designated pgec. When all three possible predicted 30 translations of the sequence were searched against protein databases such as SwissProt and PIR, no exact matches were found to the expressed protein, PGEC.

#### VI Antisense analysis

Knowledge of the correct, complete cDNA sequences of the novel expressed chemokine genes 35 will enable their use in antisense technology in the investigation of gene function. Either oligonucleotides, genomic or cDNA fragments comprising the antisense strand of lvec-1, lvec-2 or pgec can be used either *in vitro* or *in vivo* to inhibit expression of the specific protein. Such technology is now well known in the art, and probes can be designed at various locations along the nucleotide sequence. By treatment of cells or whole test animals with such antisense sequences, the gene of

interest can be effectively turned off. Frequently, the function of the gene can be ascertained by observing behavior at the cellular, tissue or organismal level (e.g. lethality, loss of differentiated function, changes in morphology or the like).

In addition to using sequences constructed to the gene itself, modifications of gene expression can be obtained by designing antisense sequences to intron regions, promoter/enhancer elements, or even to trans-acting regulatory genes. Similarly, inhibition can be achieved using Hogeboom base-pairing methodology, also known as "triple helix" base pairing.

## VII Expression of EC

The nucleotide sequences encoding the chemokines of the present invention, i.e., LVEC-1, LVEC-2 and PGEC, were cloned into an expression vector that comprises a T7 promoter followed by an initiating methionine codon (ATG), followed by six histidine codons, followed by the TrxA gene of E.coli (which encodes thioredoxin), followed by a sequence coding for an enterokinase cleavage site and the nucleotide sequences encoding the chemokine of interest. For LVEC-1, the N-terminal residue of the expressed protein is residue 20, Ala, of SEQ ID NO:2; for LVEC-2, the N-terminal residue of the expressed protein is residue 22, Gly, of SEQ ID NO:4 and for PGEC, the N-terminal residue of the expressed protein is residue 19, Gly, of SEQ ID NO:6.

Nucleotide sequences encoding PGEC (clone number 111571) were also expressed in a baculovirus system (Luckow et al. (1993) *J. Virol.* 67:4566). The N-terminal residue of the PGEC expressed in the baculovirus system was residue 20, a threonine, of SEQ ID NO: 6.

The expression vectors described above containing the 6 histidine codons were used to transform a host cell, the host cell culture was induced with IPTG and the expressed protein was subjected to denaturing SDS poly acrylamide gel electrophoresis. Nucleic acid from the expression vector was partially purified using the miniprep procedure of Sambrook *supra* which produced super-coiled DNA. About 100 ng of DNA were used to transform the host bacterial cell, W3110/DE3. W3110/DE3 was constructed using W3110 from the ATCC and the lambda DE3 lysogenization kit commercially available from Novagen. DE3 lysogens are often less competent than their parent, W3110, and are adapted to use super-coiled DNA for efficient transformation.

A single transformant from each chemokine transformation was selected and used to inoculate a 5 ml culture of L-broth containing ampicillin. Each 5 ml culture was grown overnight (12-15 hours) at 37 degrees C. with shaking. The next day, 1 ml of the overnight culture was used to inoculate a 100 ml culture of L-broth with ampicillin in a 500 ml flask and allowed to grow at 37 degrees C. with shaking until the OD600 of the culture reached 0.4-0.6. If inoculated cells are allowed to grow past an OD600 of 0.6, they will begin to reach stationary phase and induction levels will be reduced.

At the time of inoculation, a 5 ml sample was removed, placed on ice and used as a pre-induction (or 0 hour) sample. When the cell culture reached an OD600 of 0.6, 400µl of an 100mM IPTG stock solution was added for a final concentration of 0.4mM. The cultures were allowed to grow for 3 hours at 37 degrees C. with shaking. Analysis of induction was determined by sampling 5 ml aliquots of the culture at 1 hour intervals up to 6 hours and analysing on a denaturing SDS poly acrylamide gel

electrophoresis.

For the chemokines of the present invention, maximal induction occurred by 2 hours. Growth beyond 4 hours resulted in lysis in the culture and overall reduced yields of the desired protein due to proteolysis. Five ml aliquots of the cell cultures were obtained at 0, 1 and 2 hours and centrifuged for 5 minutes at 3000 RPM at 4 degrees C. The supernatant was aspirated and the pellets were subjected to a freeze-thaw step to help lyse the cells. The pellet was resuspended in TE [10mM Tris-HCL pH 8.0, 1mM EDTA pH 8.0] at 4 degrees C. at a volume calculated as:  $\text{vol TE}(\mu\text{l}) = (\text{OD600})(250)$ , and an equivalent volume of 2X SDS Sample Loading Buffer (Novex) was added to each sample. The samples were boiled for 5 minutes and 10 $\mu\text{l}$  of each sample was loaded per lane. The results of the gel electrophoresis show that LVEC-1 migrated at 25 KD molecular weight (with an expected weight of 25,347 Daltons), LVEC-2 migrated at 22 KD molecular weight (with an expected weight of 22, 378 Daltons) and PGEC migrated at 23KD molecular weight (with an expected weight of 22,879 Daltons) on a denaturing SDS gel.

#### VIII Isolation of Recombinant EC

The chemokines of the present invention were expressed as a chimeric protein having six histidines followed by the thioredoxin (TrxA) gene of E. coli with an enterokinase cleavage site between the TrxA protein and the chemokine of interest. There were 3 additional amino acid residues, GDP, between the enterokinase cleavage site and PGEC which were added to facilitate the cloning process. The histidines were added to facilitate protein purification. The presence of the histidines allows for purification on IMIAC chromatography (Porath *supra*).

#### IX Production of EC-Specific Antibodies

Polyclonal antibodies to PGEC were prepared by injecting rabbits with about 100 micrograms of electrophoresis purified PGEC fusion protein as described in Section VII. At about 8 weeks after injection of primary antigen, polyclonal antisera was collected and used in a Western Blot procedure against PGEC expressed as described in Example VII. Results of the Western Blot illustrate that the anti-PGEC polyclonal antibody is capable of binding with expressed PGEC which migrates at about 23KD on a denaturing polyacrylamide electrophoresis gel.

#### X Diagnostic Test Using EC-Specific Antibodies

Particular anti-EC antibodies are useful for the diagnosis of prepathologic conditions, and chronic or acute diseases which are characterized by differences in the amount or distribution of that EC. Particularly when an EC has only been found in the particular tissue from which it was cloned, it is likely to be specific for abnormalities or pathologies of that tissue.

Diagnostic tests for EC include methods utilizing an anti-EC antibody and a label to detect EC in human body fluids, tissues or extracts of such tissues. The polypeptides and antibodies of the present invention may be used with or without modification. Frequently, the polypeptides and antibodies will be

labeled by joining them, either covalently or noncovalently, with a substance which provides for a detectable signal. A wide variety of labels and conjugation techniques are known and have been reported extensively in both the scientific and patent literature. Suitable labels include radionuclides, enzymes, substrates, cofactors, inhibitors, fluorescent agents, chemiluminescent agents, magnetic particles and the like. Patents teaching the use of such labels include U.S. Patent Nos. 3,817,837; 3,850,752; 3,939,350; 3,996,345; 4,277,437; 4,275,149; and 4,366,241. Also, recombinant immunoglobulins may be produced as shown in U.S. Patent No. 4,816,567, incorporated herein by reference.

A variety of protocols for measuring soluble or membrane-bound EC, using either polyclonal or monoclonal antibodies specific for that EC are known in the art. Examples include enzyme-linked immunosorbent assay (ELISA), radioimmunoassay (RIA) and fluorescent activated cell sorting (FACS). A two-site monoclonal-based immunoassay utilizing monoclonal antibodies reactive to two non-interfering epitopes on EC is preferred, but a competitive binding assay may be employed. These assays are described, among other places, in Maddox, DE et al (1983) J Exp Med 158:1211.

#### XI Purification of Native EC Using Specific Antibodies

Native or recombinant ECs is purified by immunoaffinity chromatography using EC-specific antibodies. An immunoaffinity column is constructed by covalently coupling the anti-EC antibody to an activated chromatographic resin.

Polyclonal immunoglobulins are prepared as in Example IX and monoclonal antibodies are prepared from mouse ascites fluid by ammonium sulfate precipitation or chromatography on immobilized Protein A. Partially purified Ig is covalently attached to a chromatographic resin such as CnBr activated sepharose (Pharmacia LKB Biotechnology, Piscataway, NJ). The antibody is coupled to the resin, the resin is blocked, and the derivative resin is washed according to the manufacturer's instructions.

Such an immunoaffinity column is utilized in the purification of an EC of the present invention by preparing a fraction from cells containing EC in a soluble form. This preparation is derived by solubilizing of the whole cell or of a subcellular fraction obtained via differential centrifugation by the addition of detergent or by other methods well known in the art. Alternatively, soluble EC containing a signal sequence is secreted in useful quantity into the medium in which the cells are grown.

A soluble EC-containing preparation is passed over the immunoaffinity column, and the column is washed under conditions, e.g., high ionic strength buffers in the presence of detergent, that allow the preferential absorbance of EC. Then, the column is eluted under conditions that disrupt antibody/EC binding (e.g., a buffer of pH 2-3 or a high concentration of a chaotrope such as urea or thiocyanate ion), and the EC is collected.

#### XII Determination of EC-Induced Chemotaxis or Cell Activation

The chemotactic activity of EC is measured in a 48-well microchemotaxis chamber (Falk WR et al (1980) J Immunol Methods 33:239). In each well, two compartments are separated by a filter that

allows the passage of cells in response to a chemical gradient. Cell culture medium such as RPMI 1640 containing EC is placed on one side of a filter, usually polycarbonate, and cells suspended in the same media are placed on the opposite side of the filter. Sufficient incubation time is allowed for the cells to traverse the filter in response to the concentration gradient across the filter. Filters are recovered from each well, and cells adhering to the side of the filter facing EC are typed and quantified.

The specificity of the chemoattraction is determined by performing the chemotaxis assay on specific populations of cells. First, blood cells obtained from venipuncture are fractionated by density gradient centrifugation and the chemotactic activity of the particular EC is tested on enriched populations of neutrophils, peripheral blood mononuclear cells, monocytes and lymphocytes.

Optionally, such enriched cell populations are further fractionated using CD8<sup>+</sup> and CD4<sup>+</sup> specific antibodies for negative selection of CD4<sup>+</sup> and CD8<sup>+</sup> enriched T-cell populations, respectively.

Another assay elucidates the chemotactic effect of EC on activated T-cells. There, unfractionated T-cells or fractionated T-cell subsets are cultured for 6 to 8 hours in tissue culture vessels coated with CD-3 antibody. After this CD-3 activation, the chemotactic activity of EC is tested as described infra. Many other methods for obtaining enriched cell populations are known in the art.

Some chemokines also produce a non-chemotactic cell activation of neutrophils and monocytes. This is tested via standard measures of neutrophil activation such as actin polymerization, increase in respiratory burst activity, degranulation of the azurophilic granule and mobilization of Ca<sup>++</sup> as part of the signal transduction pathway. The assay for mobilization of Ca<sup>++</sup> involves preloading neutrophils with a fluorescent probe whose emission characteristics have been altered by Ca<sup>++</sup> binding. When the cells are exposed to an activating stimulus, Ca<sup>++</sup> flux is determined by observation of the cells in a fluorometer. The measurement of Ca<sup>++</sup> mobilization has been described in Grynkiewicz G et al. (1985) J Biol Chem 260:3440, and McColl S et al. (1993) J Immunol 150:4550-4555, incorporated herein by reference.

Degranulation and respiratory burst responses are also measured in monocytes (Zachariae COC et al. (1990) J Exp Med 171: 2177-82). Further measures of monocyte activation are regulation of adhesion molecule expression and cytokine production (Jiang Y et al. (1992) J Immunol 148: 2423-8). Expression of adhesion molecules also varies with lymphocyte activation (Taub D et al. (1993) Science 260: 355-358).

Chemically synthesized PGEC having the N-terminal residue Threonine (amino acid residue 20 of SEQ ID NO:6) was found to induce the chemotaxis of THP-1 human monocytes in vitro using the classical Boyden chamber. As illustrated in Figure 12, chemotaxis of THP-1 cells (ATCC accession number TIB 202) was measured in response to varying concentrations of PGEC, (Incyte clone number 111571). The results show an IC<sub>50</sub> of approximately 100nM.

### XIII Drug Screening

This ECs of the present invention, or fragments thereof, are particularly useful for screening compounds in any of a variety of drug screening techniques. The EC polypeptide or fragment employed

in such a test is either free in solution, affixed to a solid support, borne on a cell surface or located intracellularly. One method of drug screening utilizes eukaryotic or prokaryotic host cells which are stably transformed with recombinant nucleic acids expressing the EC polypeptide or fragment thereof. Drugs are screened against such transformed cells in competitive binding assays. Such cells, either in  
5 viable or fixed form, are used for standard binding assays. One may measure, for example, the formation of complexes between an EC polypeptide or fragment and the agent being tested or examine the diminution in complex formation between a EC polypeptide or fragment and cell caused by the agent being tested.

Thus, the present invention provides methods of screening for drugs or any other agents which  
10 can affect inflammation and disease. These methods comprise contacting such an agent with a EC polypeptide of the present invention, or fragment thereof, and assaying (i) for the presence of a complex between the agent and the EC polypeptide or fragment, or (ii) for the presence of a complex between the EC polypeptide or fragment and the cell, by methods well known in the art. In such competitive binding assays, the EC polypeptide or fragment is labeled by methods known to those of  
15 skill in the art. After suitable incubation, free EC polypeptide or fragment is separated from that present in bound form, and the amount of free or uncomplexed label is a measure of the ability of the particular agent to bind to the EC, or fragment thereof, or to interfere with the EC/cell complex.

Another technique for drug screening provides high throughput screening for compounds having suitable binding affinity to the EC polypeptides of the present invention, or fragments thereof, and is  
20 described in detail in Geysen, European Patent Application 84/03564, published on September 13, 1984, incorporated herein by reference. Briefly stated, large numbers of different small peptide test compounds are synthesized on a solid substrate, such as plastic pins or some other surface. The peptide test compounds are reacted with EC polypeptide and washed. Bound EC polypeptide is then detected by methods well known in the art. Purified EC can also be coated directly onto plates for use  
25 in the aforementioned drug screening techniques. In addition, non-neutralizing antibodies can be used to capture the peptide and immobilize it on the solid support.

This invention also contemplates the use of competitive drug screening assays in which neutralizing antibodies capable of binding EC specifically compete with a test compound for binding to EC polypeptide or fragments thereof. In this manner, the antibodies can be used to detect the presence of  
30 any peptide which shares one or more antigenic determinants with EC.

#### **XIV Rational Drug Design**

The goal of rational drug design is to produce structural analogs of biologically active polypeptides of interest or of small molecules with which they interact, e.g., agonists, antagonists, or  
35 inhibitors. Any of these examples can be used to fashion drugs which are more active or stable forms of the polypeptide or which enhance or interfere with the function of a polypeptide *in vivo* (cf. Hodgson J (1991) Bio/Technology 9:19-21, incorporated herein by reference).

In one approach, the three-dimensional structure of a protein of interest, or of a protein-inhibitor complex, is determined by x-ray crystallography, by computer modeling or, most typically,

by a combination of the two approaches. Both the shape and charges of the polypeptide must be ascertained to elucidate the structure and to determine active site(s) of the molecule. Useful information regarding the structure of a polypeptide may be gained by modeling based on the structure of homologous proteins. In both cases, relevant structural information is used to design analogous chemokine-like molecules or to identify efficient inhibitors. Useful examples of rational drug design may include molecules which have improved EC activity or stability as shown by Braxton S and Wells JA (1992 Biochemistry 31:7796-7801) or which act as inhibitors, agonists, or antagonists of naturally occurring EC as shown by Athauda SB et al (1993 J Biochem 113:742-746), incorporated herein by reference.

It is also possible to isolate a target-specific antibody, selected by functional assay, as described *infra*, and then to solve its crystal structure. This approach, in principle, yields a pharmacore upon which subsequent drug design can be based. It is possible to bypass protein crystallography altogether by generating anti-idiotypic antibodies (anti-ids) to a functional, pharmacologically active antibody. As a mirror image of a mirror image, the binding site of the anti-ids would be expected to be an analog of the original receptor. The anti-id could then be used to identify and isolate peptides from banks of chemically or biologically produced peptides. The isolated peptides would then act as the pharmacore.

Using methods disclosed *infra*, sufficient amount of an EC polypeptide may be made available to perform analytical studies such as X-ray crystallography. In addition, knowledge of the EC amino acid sequence disclosed herein will provide guidance to those employing computer modeling techniques in place of or in addition to x-ray crystallography.

#### **XV Identification of EC Receptors**

A purified EC of the present invention, or fragment thereof, can be used to characterize and purify specific cell surface receptors and other binding molecules. Cells which respond to a particular EC by chemotaxis or other specific responses are likely to express a receptor for that EC. Radioactive labels are incorporated into ECs of the present invention, or fragments thereof, by various methods known to those of skill in the art. A preferred embodiment is the labeling of primary amino groups in EC with <sup>125</sup>I Bolton-Hunter reagent (Bolton, AE and Hunter, WM (1973) Biochem J 133: 529), which has been used to label other chemokines without concomitant loss of biological activity (Hebert CA et al (1991) J Biol Chem 266: 18989; McColl S et al (1993) J Immunol 150:4550-4555). Receptor-bearing cells are incubated with labeled EC. The cells are then washed to removed unbound EC, and receptor-bound EC is quantified. The data obtained using different concentrations of EC are used to calculate values for the number and affinity of receptors.

Labeled EC is useful as a reagent for purification of its specific receptor. In one embodiment of affinity purification, the EC is covalently coupled to a chromatography column. Receptor-bearing cells are extracted, and the extract is passed over the column. The receptor binds to the column by virtue of its biological affinity for its ligand. The receptor is recovered from the column and subjected to N-terminal protein sequencing. This amino acid sequence is then used to design degenerate oligonucleotide



probes for cloning the receptor gene.

In an alternate method, expression cloning, mRNA is obtained from receptor-bearing cells and made into a cDNA expression library. The library is transfected into a population of cells, and those cells in the population which express the receptor are selected using fluorescently labeled EC. The receptor is identified by recovering and sequencing recombinant DNA from highly labeled cells.

In another alternate method, antibodies, preferably monoclonal antibodies, are raised against the surface of receptor-bearing cells. The monoclonal antibodies are screened to identify those which inhibit the binding of labeled EC. These monoclonal antibodies are then used in affinity purification or expression cloning of the receptor.

Soluble receptors or other soluble binding molecules are identified in a similar manner. Labeled ECs are incubated with extracts or other appropriate materials derived from their specific inflamed or diseased tissue. After incubation, EC complexes larger than the size of the purified EC are identified by a sizing technique, such as, for example, size exclusion chromatography or density gradient centrifugation, and are purified by methods known in the art. The soluble receptors or binding protein(s) are subjected to N-terminal sequencing to obtain information sufficient for database identification, if the soluble protein is known, or cloning, if the soluble protein is unknown.

Chemically synthesized PGEC having the N-terminal amino acid residue Threonine (amino acid residue 20 of SEQ ID NO:6) was found to induce a calcium response on THP-1 human monocytes. The receptor utilization for the calcium response and chemotaxis in THP-1 cells (illustrated in Figure 12) was characterized using cross desensitization experiments taking advantage of the normal receptor downregulation that occurs after agonist ligation.

Figure 11 demonstrates the change in free intracellular calcium in response to PGEC (labeled 111571) in THP-1 cells (ATCC TIB 202). Intracellular calcium measurements were done as described in Naccache et al.(1989) *J. Immunol.* 142:2438-2444) and Neote et al.(1993) *Cell* 72:415-425). Cells were loaded with the calcium probe INDO-1-AM at 37 degrees C. in 1X HANKS and assayed in the same buffer spectrofluorometrically as described.

Figure 13 demonstrates the effects of PGEC (labeled 111571) and MIP-1 alpha in THP-1 cells. Figure 13 demonstrates that a high concentration of PGEC elicits a response and blocks THP-1 response to MIP-1 $\alpha$  whereas the response to MCP-1 is not affected. Administration of MIP-1 $\alpha$  essentially prohibited PGEC response but not the MCP-1 response. Given the array of chemokine receptors expressed on THP-1 cells, this observation suggests that PGEC acts via the CC chemokine receptor designated CC-CKR-1(Neote et al. (1993) *Cell* 72:415-425).

As demonstrated in Figure 11, the IC<sub>50</sub> in the calcium flux assay is about 120 nM and is consistent with the IC<sub>50</sub> value obtained in the chemotaxis assay demonstrated in Figure 12. The data suggest that PGEC has a low affinity interaction with CC-CKR-1.

All publications and patents mentioned in the above specification are herein incorporated by reference. The foregoing written specification is considered to be sufficient to enable one skilled in the art to practice the invention. Indeed, various modifications of the above described modes for carrying out the invention which are obvious to those skilled in the field of molecular biology or related fields

are intended to be within the scope of the following claims.

5

10

15

## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

- (i) APPLICANT: INCYTE PHARMACEUTICALS, INC.
- (ii) TITLE OF INVENTION: EXPRESSED CHEMOKINES, THEIR PRODUCTION AND USES
- (iii) NUMBER OF SEQUENCES: 6
- (iv) CORRESPONDENCE ADDRESS:
  - (A) ADDRESSEE: INCYTE PHARMACEUTICALS, INC.
  - (B) STREET: 3174 PORTER DRIVE
  - (C) CITY: PALO ALTO
  - (D) STATE: CA
  - (E) COUNTRY: USA
  - (F) ZIP: 94304
- (v) COMPUTER READABLE FORM:
  - (A) MEDIUM TYPE: Floppy disk
  - (B) COMPUTER: IBM PC compatible
  - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
  - (D) SOFTWARE: PatentIn Release #1.0, Version #1.30
- (vi) CURRENT APPLICATION DATA:
  - (A) PCT APPLICATION NO. To Be Assigned
  - (B) FILING DATE: 29-NOV-1995
  - (C) CLASSIFICATION:
- (vii) PRIOR APPLICATION DATA:
  - (A) APPLICATION SERIAL NO: US 08/347,492
  - (B) FILING DATE: 29-NOV-1994
  - (C) CLASSIFICATION:
- (viii) ATTORNEY/AGENT INFORMATION:
  - (A) NAME: LUTHER, BARBARA J
  - (B) REGISTRATION NUMBER: 33954
  - (C) REFERENCE/DOCKET NUMBER: PF-0024 PCT
- (ix) TELECOMMUNICATION INFORMATION:
  - (A) TELEPHONE: 415-855-0555
  - (B) TELEFAX: 415-852-0195

## (2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 363 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: cDNA  
 (vii) IMMEDIATE SOURCE:  
     (A) LIBRARY: Human Liver  
     (B) CLONE: 87825

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

```

ATGAAGGTCT CCGAGGCTGC CCTGTCTCTC CTTGTCCTCA TCCTTATCAT TACTTCGGCT      60
TCTCGCAGCC AGCCAAAAGT TCCTGAGTGG GTGAACACCC CATCCACCTG CTGCCTGAAG      120
TATTATGAGA AAGTGTTGCC AAGGAGACTA GTGGTGGGAT ACAGAAAGGC CCTCAACTGT      180
CACCTGCCAG CAATCATCTT CGTCACCAAG AGGAACCGAG AAGTCTGCAC CAACCCCAAT      240
GACGACTGGG TCCAAGAGTA CATCAAGGAT CCCAACCTAC CTTTGCTGCC TACCAGGAAC      300
TTGTCCACGG TTAAAATTAT TACAGCAAAG AATGGTCAAC CCCAGCTCCT CAACTCCCAG      360
TGA                                                                    363
  
```

## (2) INFORMATION FOR SEQ ID NO:2:

- (i) SEQUENCE CHARACTERISTICS:  
     (A) LENGTH: 120 amino acids  
     (B) TYPE: amino acid  
     (C) STRANDEDNESS: single  
     (D) TOPOLOGY: unknown
- (ii) MOLECULE TYPE: peptide
- (vii) IMMEDIATE SOURCE:  
     (A) LIBRARY: Liver  
     (B) CLONE: 87825
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:
- ```

Met Lys Val Ser Glu Ala Ala Leu Ser Leu Leu Val Leu Ile Leu Ile
1           5           10          15
Ile Thr Ser Ala Ser Arg Ser Gln Pro Lys Val Pro Glu Trp Val Asn
20          25          30
Thr Pro Ser Thr Cys Cys Leu Lys Tyr Tyr Glu Lys Val Leu Pro Arg
35          40          45
Arg Leu Val Val Gly Tyr Arg Lys Ala Leu Asn Cys His Leu Pro Ala
50          55          60
  
```

Ile Ile Phe Val Thr Lys Arg Asn Arg Glu Val Cys Thr Asn Pro Asn  
 65 70 75 80

Asp Asp Trp Val Gln Glu Tyr Ile Lys Asp Pro Asn Leu Pro Leu Leu  
 85 90 95

Pro Thr Arg Asn Leu Ser Thr Val Lys Ile Ile Thr Ala Lys Asn Gly  
 100 105 110

Gln Pro Gln Leu Leu Asn Ser Gln  
 115 120

## (2) INFORMATION FOR SEQ ID NO:3:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 291 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: cDNA

## (vii) IMMEDIATE SOURCE:

- (A) LIBRARY: Liver
- (B) CLONE: 88564

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

ATGTGCTGTA CCAAGAGTTT GTCCTGGCT GCTTTGATGT CAGTGCTGCT ACTCCACCTC 60

TGCGGCGAAT CAGAAGCAGC AAGCAACTTT GACTGCTGTC TTGGATACAC AGACCGTATT 120

CTTCATCCTA AATTTATTGT GGGCTTCACA CGGCAGCTGG CCAATGAAGG CTGTGACATC 180

AATGCTATCA TCTTTCACAC AAAGAAAAAG TTGTCTGTGT GCGCAAATCC AAAACAGACT 240

TGGGTGAAAT ATATTGTGCG TCTCCTCAGT AAAAAAGTCA AGAACATGTA A 291

## (2) INFORMATION FOR SEQ ID NO:4:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 96 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide

(vii) IMMEDIATE SOURCE:

(A) LIBRARY: Human Liver

(B) CLONE: 88564

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

```

Met Cys Cys Thr Lys Ser Leu Leu Leu Ala Ala Leu Met Ser Val Leu
 1             5             10             15

Leu Leu His Leu Cys Gly Glu Ser Glu Ala Ala Ser Asn Phe Asp Cys
      20             25             30

Cys Leu Gly Tyr Thr Asp Arg Ile Leu His Pro Lys Phe Ile Val Gly
      35             40             45

Phe Thr Arg Gln Leu Ala Asn Glu Gly Cys Asp Ile Asn Ala Ile Ile
 50             55             60

Phe His Thr Lys Lys Lys Leu Ser Val Cys Ala Asn Pro Lys Gln Thr
 65             70             75             80

Trp Val Lys Tyr Ile Val Arg Leu Leu Ser Lys Lys Val Lys Asn Met
      85             90             95

```

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 282 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(vii) IMMEDIATE SOURCE:

(A) LIBRARY: Pituitary Gland

(B) CLONE: 111571

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

```

ATGAAGATCT CCGTGGCTGC CATTCCCTTC TTCCTCCTCA TCACCATCGC CCTAGGGACC      60

AAGACTGAAT CCTCCTCACG GGGACCTTAC CACCCCTCAG AGTGCTGCTT CACCTACACT      120

ACCTACAAGA TCCCGCGTCA GCGGATTATG GATTACTATG AGACCAACAG CCAGTGCTCC      180

AAGCCCGGAA TTGTCTTCAT CACCAAAAGG GGCCATTCCG TCTGTACCAA CCCCAGTGAC      240

```

AAGTGGGTCC AGGACTATAT CAAGGACATG AAGGAGAACT GA

282

## (2) INFORMATION FOR SEQ ID NO:6:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 93 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: unknown

## (ii) MOLECULE TYPE: peptide

## (vii) IMMEDIATE SOURCE:

- (A) LIBRARY: Pituitary gland
- (B) CLONE: 111571

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

|     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| Met | Lys | Ile | Ser | Val | Ala | Ala | Ile | Pro | Phe | Phe | Leu | Leu | Ile | Thr | Ile |
| 1   |     |     |     | 5   |     |     |     | 10  |     |     |     |     |     | 15  |     |
| Ala | Leu | Gly | Thr | Lys | Thr | Glu | Ser | Ser | Ser | Arg | Gly | Pro | Tyr | His | Pro |
|     |     |     | 20  |     |     |     |     | 25  |     |     |     |     | 30  |     |     |
| Ser | Glu | Cys | Cys | Phe | Thr | Tyr | Thr | Thr | Tyr | Lys | Ile | Pro | Arg | Gln | Arg |
|     |     | 35  |     |     |     |     |     | 40  |     |     |     |     | 45  |     |     |
| Ile | Met | Asp | Tyr | Tyr | Glu | Thr | Asn | Ser | Gln | Cys | Ser | Lys | Pro | Gly | Ile |
|     | 50  |     |     |     |     | 55  |     |     |     |     | 60  |     |     |     |     |
| Val | Phe | Ile | Thr | Lys | Arg | Gly | His | Ser | Val | Cys | Thr | Asn | Pro | Ser | Asp |
| 65  |     |     |     |     | 70  |     |     |     |     | 75  |     |     |     | 80  |     |
| Lys | Trp | Val | Gln | Asp | Tyr | Ile | Lys | Asp | Met | Lys | Glu | Asn |     |     |     |
|     |     |     | 85  |     |     |     |     |     | 90  |     |     |     |     |     |     |

**CLAIMS**

- 5           1. A purified polynucleotide comprising a polynucleotide sequence encoding the polypeptide having the sequence as depicted in SEQ ID NO: 2, SEQ ID NO: 4 or SEQ ID NO: 6, or its complement.
2. The polynucleotide of Claim 1 wherein the polynucleotide sequence consists of SEQ ID NO: 1, SEQ ID NO: 3 or SEQ ID NO: 5.
- 10           3. A purified polynucleotide capable of hybridizing to the polynucleotide of Claim 2 under stringent conditions.
4. An expression vector comprising the purified polynucleotide of Claim 2.
- 15           5. A host cell comprising the expression vector of Claim 4.
6. A polynucleotide probe comprising a non-conserved fragment of the polynucleotide of Claim 2.
- 20           7. An antisense molecule comprising a polynucleotide sequence complementary to at least a portion of the polynucleotide of Claim 2.
8. A method for producing a polypeptide comprising the sequence as depicted in SEQ ID NO:2, SEQ ID NO:4, or SEQ ID NO:6, said method comprising:
- 25           a) culturing the host cells of Claim 5 under conditions suitable for the expression of the polypeptide, and
- b) recovering said polypeptide from the cell culture.
- 30           9. A diagnostic test for the detection of nucleotide sequences encoding liver expressed chemokine 1 in a biological sample, comprising the steps of:
- a) combining the biological sample with a first nucleotide sequence which comprises the nucleotide sequence of SEQ ID NO:1, or a fragment thereof, under conditions suitable for the formation
- 35           of a nucleic acid hybridization complex,
- b) detecting said hybridization complex, wherein the presence of said complex correlates with the presence of a second nucleotide sequence encoding liver expressed chemokine 1 in said biological sample, and
- c) comparing the amount of the second nucleotide sequence in said sample with a standard
- 40           thereby determining whether the amount of said second nucleotide sequence varies from said standard, wherein the presence of an abnormal level of said second nucleotide sequence correlates positively with a condition associated with inflammation of the liver.
10. A diagnostic test for the detection of nucleotide sequences encoding liver expressed chemokine 2 in a biological sample, comprising the steps of:
- 45           a) combining the biological sample with a first nucleotide sequence which comprises the nucleotide sequence of SEQ ID NO:3, or a fragment thereof, under conditions suitable for the formation of a nucleic acid hybridization complex,
- b) detecting said hybridization complex, wherein the presence of said complex correlates with
- 50           the presence of a second nucleotide sequence encoding liver expressed chemokine 2 in said biological sample, and
- c) comparing the amount of the second nucleotide sequence in said sample with a standard thereby determining whether the amount of said second nucleotide sequence varies from said standard, wherein the presence of an abnormal level of said second nucleotide sequence correlates
- 55           positively with a condition associated with inflammation.



11. A diagnostic test for the detection of nucleotide sequences encoding pituitary gland expressed chemokine in a biological sample, comprising the steps of:

a) combining the biological sample with a first nucleotide sequence which comprises the nucleotide sequence of SEQ ID NO:5, or a fragment thereof, under conditions suitable for the formation of a nucleic acid hybridization complex,

b) detecting said hybridization complex, wherein the presence of said complex correlates with the presence of a second nucleotide sequence encoding pituitary gland expressed chemokine in said biological sample, and

c) comparing the amount of the second nucleotide sequence in said sample with a standard thereby determining whether the amount of said second nucleotide sequence varies from said standard, wherein the presence of an abnormal level of said second nucleotide sequence correlates positively with a condition associated with inflammation.

12. The diagnostic test of Claim 9, Claim 10 or Claim 11 wherein said first nucleotide sequence is labeled with a reporter molecule and the hybridization complex is detected by measuring said reporter molecule.

13. A diagnostic test for the detection of nucleotide sequences encoding liver expressed chemokine 1 in a biological sample, comprising the steps of:

a) combining the biological sample with polymerase chain reaction primers under conditions suitable for nucleic acid amplification, wherein said primers comprise fragments of the nucleotide sequence of SEQ ID NO:1,

c) detecting amplified nucleotide sequences, and

d) comparing the amount of amplified nucleotide sequences in said biological sample with a standard thereby determining whether the amount of said nucleotide sequence varies from said standard, wherein the presence of an abnormal level of said nucleotide sequence correlates positively with a condition associated with inflammation of the liver.

14. A diagnostic test for the detection of nucleotide sequences encoding liver expressed chemokine 2 in a biological sample, comprising the steps of:

a) combining the biological sample with polymerase chain reaction primers under conditions suitable for nucleic acid amplification, wherein said primers comprise fragments of the nucleotide sequence of SEQ ID NO:3,

c) detecting amplified nucleotide sequences, and

d) comparing the amount of amplified nucleotide sequences in said biological sample with a standard thereby determining whether the amount of said nucleotide sequence varies from said standard, wherein the presence of an abnormal level of said nucleotide sequence correlates positively with a condition associated with inflammation.

15. A diagnostic test for the detection of nucleotide sequences encoding pituitary gland expressed chemokine in a biological sample, comprising the steps of:

a) combining the biological sample with polymerase chain reaction primers under conditions suitable for nucleic acid amplification, wherein said primers comprise fragments of the nucleotide sequence of SEQ ID NO:5,

c) detecting amplified nucleotide sequences, and

d) comparing the amount of amplified nucleotide sequences in said biological sample with a standard thereby determining whether the amount of said nucleotide sequence varies from said standard, wherein the presence of an abnormal level of said nucleotide sequence correlates positively with a condition associated with inflammation.

16. A purified polypeptide wherein the polypeptide sequence consists of SEQ ID NO: 2, SEQ ID NO: 4 or SEQ ID NO: 6.

17. An antibody specific for the purified polypeptide of Claim 16.

18. Purified PGEC having an N-terminal amino acid residue of residue 20, threonine, of SEQ ID  
NO: 6.
- 5 19. Purified PGEC having an N-terminal amino acid residue of residue 19, glycine, of SEQ ID  
NO: 6.
20. Purified LVEC-1 having an N-terminal amino acid residue of residue 20, alanine, of SEQ ID  
NO: 2.
- 10 21. Purified LVEC-2 having an N-terminal amino acid residue of residue 22, glycine, of SEQ ID  
NO: 4.
22. The use of the purified PGEC of Claim 18 to selectively affect the CC-CKR1 receptor over  
the MCP-1 receptor.

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```

      9      18      27      36      45      54
5' ATG AAG GTC TCC GAG GCT GCC CTG TCT CTC CTT GTC CTC ATC CTT ATC ATT ACT
   M  K  V  S  E  A  A  L  S  L  L  V  L  I  L  I  I  T

      63      72      81      90      99      108
   TCG GCT TCT CGC AGC CAG CCA AAA GTT CCT GAG TGG GTG AAC ACC CCA TCC ACC
   S  A  S  R  S  Q  P  K  V  P  E  W  V  N  T  P  S  T

      117      126      135      144      153      162
   TGC TGC CTG AAG TAT TAT GAG AAA GTG TTG CCA AGG AGA CTA GTG GTG GGA TAC
   C  C  L  K  Y  Y  E  K  V  L  P  R  R  L  V  V  G  Y

      171      180      189      198      207      216
   AGA AAG GCC CTC AAC TGT CAC CTG CCA GCA ATC ATC TTC GTC ACC AAG AGG AAC
   R  K  A  L  N  C  H  L  P  A  I  I  F  V  T  K  R  N

      225      234      243      252      261      270
   CGA GAA GTC TGC ACC AAC CCC AAT GAC GAC TGG GTC CAA GAG TAC ATC AAG GAT
   R  E  V  C  T  N  P  N  D  D  W  V  Q  E  Y  I  K  D

      279      288      297      306      315      324
   CCC AAC CTA CCT TTG CTG CCT ACC AGG AAC TTG TCC ACG GTT AAA ATT ATT ACA
   P  N  L  P  L  L  P  T  R  N  L  S  T  V  K  I  I  T

      333      342      351      360
   GCA AAG AAT GGT CAA CCC CAG CTC CTC AAC TCC CAG TGA 3'
   A  K  N  G  Q  P  Q  L  L  N  S  Q  *

```

FIGURE 1

Predicted Structural Class of the Whole Protein: Alpha  
Deléage & Roux Modification of Nishikawa & Ooi 1987

| Analysis                     | Whole Protein |
|------------------------------|---------------|
| Molecular Weight             | 13600.30 m.w. |
| Length                       | 120           |
| 1 microgram =                | 73.528 pMoles |
| Molar Extinction coefficient | 16980±5%      |
| 1 A(280) =                   | 0.80 mg/ml    |
| Isoelectric Point            | 9.57          |
| Charge at pH 7               | 7.96          |

Whole Protein Composition Analysis

| Amino Acid(s)        | Number<br>count | % by<br>weight | % by<br>frequency |
|----------------------|-----------------|----------------|-------------------|
| Charged (RKHYCDE)    | 33              | 32.65          | 27.50             |
| Acidic (DE)          | 8               | 7.29           | 6.67              |
| Basic (KR)           | 16              | 16.52          | 13.33             |
| Polar (NCQSTY)       | 38              | 31.17          | 31.67             |
| Hydrophobic (AILFWV) | 44              | 34.95          | 36.67             |
| A Ala                | 6               | 3.14           | 5.00              |
| C Cys                | 4               | 3.04           | 3.33              |
| D Asp                | 3               | 2.54           | 2.50              |
| E Glu                | 5               | 4.75           | 4.17              |
| F Phe                | 1               | 1.08           | 0.83              |
| G Gly                | 2               | 0.84           | 1.67              |
| H His                | 1               | 1.01           | 0.83              |
| I Ile                | 8               | 6.66           | 6.67              |
| K Lys                | 9               | 8.48           | 7.50              |
| L Leu                | 16              | 13.32          | 13.33             |
| M Met                | 1               | 0.96           | 0.83              |
| N Asn                | 9               | 7.55           | 7.50              |
| P Pro                | 10              | 7.14           | 8.33              |
| Q Gln                | 5               | 4.71           | 4.17              |
| R Arg                | 7               | 8.04           | 5.83              |
| S Ser                | 8               | 5.12           | 6.67              |
| T Thr                | 8               | 5.95           | 6.67              |
| V Val                | 11              | 8.02           | 9.17              |
| W Trp                | 2               | 2.74           | 1.67              |
| Y Tyr                | 4               | 4.80           | 3.33              |
| B Asx                | 0               | 0.00           | 0.00              |
| Z Glx                | 0               | 0.00           | 0.00              |
| X Xxx                | 0               | 0.00           | 0.00              |
| . Ter                | 0               | 0.00           | 0.00              |

FIGURE 2

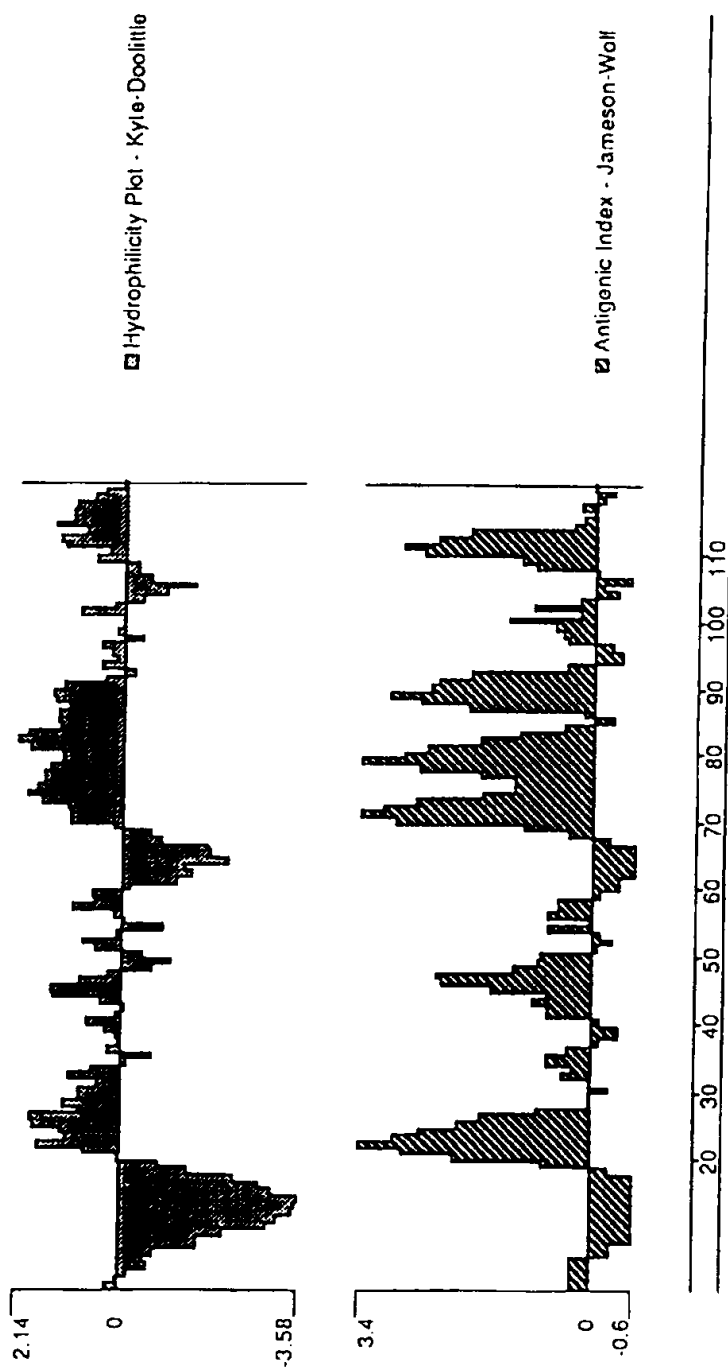


FIGURE 3

```

      9      18      27      36      45      54
5' ATG TGC TGT ACC AAG AGT TTG CTC CTG GCT GCT TTG ATG TCA GTG CTG CTA CTC
   M  C  C  T  K  S  L  L  L  A  A  L  M  S  V  L  L  L

      63      72      81      90      99      108
CAC CTC TGC GGC GAA TCA GAA GCA GCA AGC AAC TTT GAC TGC TGT CTT GGA TAC
H  L  C  G  E  S  E  A  A  S  N  F  D  C  C  L  G  Y

      117      126      135      144      153      162
ACA GAC CGT ATT CTT CAT CCT AAA TTT ATT GTG GGC TTC ACA CGG CAG CTG GCC
T  D  R  I  L  H  P  K  F  I  V  G  F  T  R  Q  L  A

      171      180      189      198      207      216
AAT GAA GGC TGT GAC ATC AAT GCT ATC ATC TTT CAC ACA AAG AAA AAG TTG TCT
N  E  G  C  D  I  N  A  I  I  F  H  T  K  K  K  L  S

      225      234      243      252      261      270
GTG TGC GCA AAT CCA AAA CAG ACT TGG GTG AAA TAT ATT GTG CGT CTC CTC AGT
V  C  A  N  P  K  Q  T  W  V  K  Y  I  V  R  L  L  S

      279      288
AAA AAA GTC AAG AAC ATG TAA 3'
K  K  V  K  N  M  *

```

FIGURE 4

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Predicted Structural Class of the Whole Protein: Alpha  
 Deléage & Roux Modification of Nishikawa & Ooi 1987

| Analysis                     | Whole Protein |
|------------------------------|---------------|
| Molecular Weight             | 10762.90 m.w. |
| Length                       | 96            |
| 1 microgram =                | 92.912 pMoles |
| Molar Extinction coefficient | 9090±5%       |
| 1 A(280) =                   | 1.18 mg/ml    |
| Isoelectric Point            | 8.95          |
| Charge at pH 7               | 7.19          |

## Whole Protein Composition Analysis

| Amino Acid(s)        | Number<br>count | % by<br>weight | % by<br>frequency |
|----------------------|-----------------|----------------|-------------------|
| Charged (RKHYCDE)    | 31              | 36.64          | 32.29             |
| Acidic (DE)          | 6               | 6.81           | 6.25              |
| Basic (KR)           | 13              | 16.27          | 13.54             |
| Polar (NCQSTY)       | 27              | 26.98          | 28.12             |
| Hydrophobic (AILFWV) | 38              | 38.38          | 39.58             |
| A Ala                | 7               | 4.62           | 7.29              |
| C Cys                | 7               | 6.71           | 7.29              |
| D Asp                | 3               | 3.21           | 3.12              |
| E Glu                | 3               | 3.60           | 3.12              |
| F Phe                | 4               | 5.47           | 4.17              |
| G Gly                | 4               | 2.12           | 4.17              |
| H His                | 3               | 3.82           | 3.12              |
| I Ile                | 6               | 6.31           | 6.25              |
| K Lys                | 10              | 11.91          | 10.42             |
| L Leu                | 14              | 14.72          | 14.58             |
| M Met                | 3               | 3.66           | 3.12              |
| N Asn                | 5               | 5.30           | 5.21              |
| P Pro                | 2               | 1.80           | 2.08              |
| Q Gln                | 2               | 2.38           | 2.08              |
| R Arg                | 3               | 4.35           | 3.12              |
| S Ser                | 6               | 4.86           | 6.25              |
| T Thr                | 5               | 4.70           | 5.21              |
| V Val                | 6               | 5.52           | 6.25              |
| W Trp                | 1               | 1.73           | 1.04              |
| Y Tyr                | 2               | 3.03           | 2.08              |
| B Asx                | 0               | 0.00           | 0.00              |
| Z Glx                | 0               | 0.00           | 0.00              |
| X Xxx                | 0               | 0.00           | 0.00              |
| . Ter                | 0               | 0.00           | 0.00              |

FIGURE 5

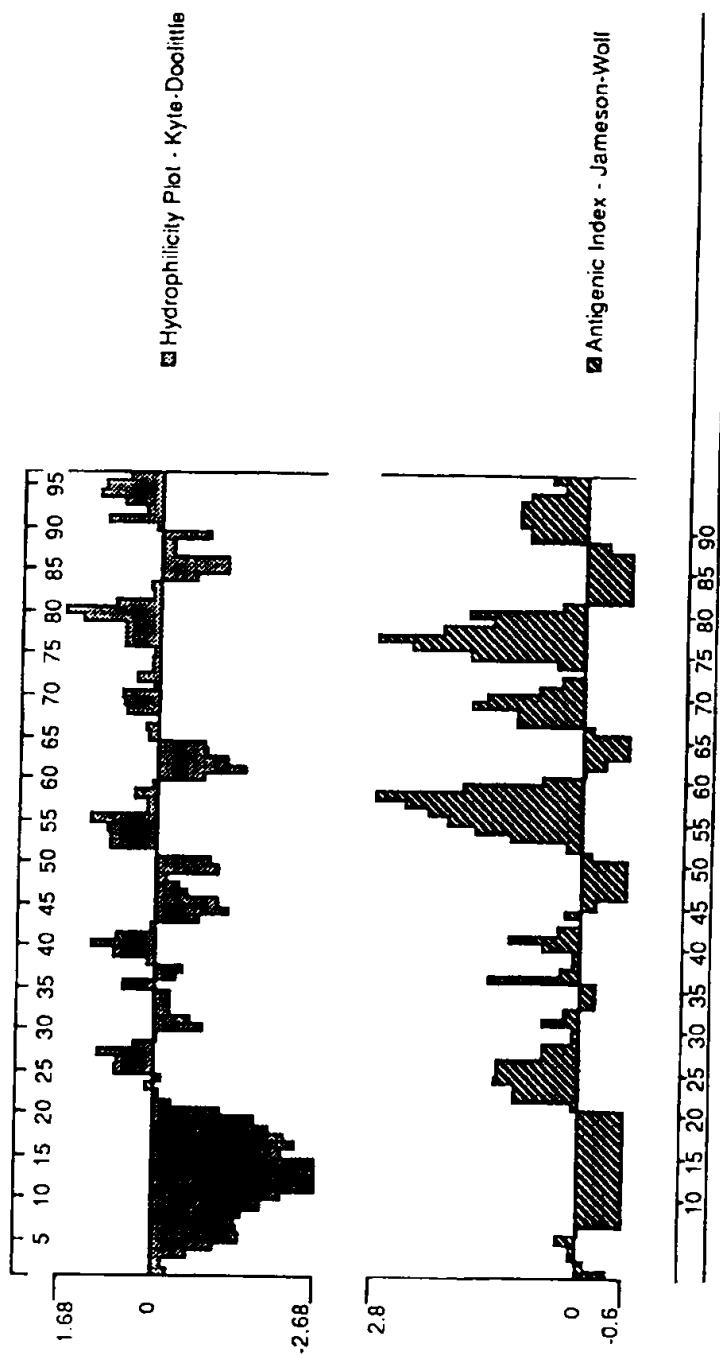


FIGURE 6



```

      9      18      27      36      45      54
5' ATG AAG ATC TCC GTG GCT GCC ATT CCC TTC TTC CTC CTC ATC ACC ATC GCC CTA
   M  K  I  S  V  A  A  I  P  F  F  L  L  I  T  I  A  L

      63      72      81      90      99      108
   GGG ACC AAG ACT GAA TCC TCC TCA CGG GGA CCT TAC CAC CCC TCA GAG TGC TGC
   G  T  K  T  E  S  S  S  R  G  P  Y  H  P  S  E  C  C

      117      126      135      144      153      162
   TTC ACC TAC ACT ACC TAC AAG ATC CCG CGT CAG CGG ATT ATG GAT TAC TAT GAG
   F  T  Y  T  T  Y  K  I  P  R  Q  R  I  M  D  Y  Y  E

      171      180      189      198      207      216
   ACC AAC AGC CAG TGC TCC AAG CCC GGA ATT GTC TTC ATC ACC AAA AGG GGC CAT
   T  N  S  Q  C  S  K  P  G  I  V  F  I  T  K  R  G  H

      225      234      243      252      261      270
   TCC GTC TGT ACC AAC CCC AGT GAC AAG TGG GTC CAG GAC TAT ATC AAG GAC ATG
   S  V  C  T  N  P  S  D  K  W  V  Q  D  Y  I  K  D  M

      279
   AAG GAG AAC TGA 3'
   K  E  N  *

```

FIGURE 7

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Predicted Structural Class of the Whole Protein: Alpha  
Deléage & Roux Modification of Nishikawa & Ooi 1987

| Analysis                     | Whole Protein |
|------------------------------|---------------|
| Molecular Weight             | 10678.70 m.w. |
| Length                       | 93            |
| 1 microgram =                | 93.644 pMoles |
| Molar Extinction coefficient | 13850±5%      |
| 1 A(280) =                   | 0.77 mg/ml    |
| Isoelectric Point            | 8.73          |
| Charge at pH 7               | 4.12          |

## Whole Protein Composition Analysis

| Amino Acid(s)        | Number<br>count | % by<br>weight | % by<br>frequency |
|----------------------|-----------------|----------------|-------------------|
| Charged (RKHYCDE)    | 32              | 40.21          | 34.41             |
| Acidic (DE)          | 8               | 9.15           | 8.60              |
| Basic (KR)           | 12              | 15.46          | 12.90             |
| Polar (NCQSTY)       | 34              | 35.70          | 36.56             |
| Hydrophobic (AILFWV) | 24              | 25.69          | 25.81             |
| A Ala                | 3               | 2.00           | 3.23              |
| C Cys                | 4               | 3.87           | 4.30              |
| D Asp                | 4               | 4.31           | 4.30              |
| E Glu                | 4               | 4.84           | 4.30              |
| F Phe                | 4               | 5.51           | 4.30              |
| G Gly                | 4               | 2.14           | 4.30              |
| H His                | 2               | 2.57           | 2.15              |
| I Ile                | 9               | 9.54           | 9.68              |
| K Lys                | 8               | 9.60           | 8.60              |
| L Leu                | 3               | 3.18           | 3.23              |
| M Met                | 3               | 3.69           | 3.23              |
| N Asn                | 3               | 3.21           | 3.23              |
| P Pro                | 6               | 5.46           | 6.45              |
| Q Gln                | 3               | 3.60           | 3.23              |
| R Arg                | 4               | 5.85           | 4.30              |
| S Ser                | 9               | 7.34           | 9.68              |
| T Thr                | 9               | 8.52           | 9.68              |
| V Val                | 4               | 3.71           | 4.30              |
| W Trp                | 1               | 1.74           | 1.08              |
| Y Tyr                | 6               | 9.17           | 6.45              |
| B Asx                | 0               | 0.00           | 0.00              |
| Z Glx                | 0               | 0.00           | 0.00              |
| X Xxx                | 0               | 0.00           | 0.00              |
| . Ter                | 0               | 0.00           | 0.00              |

FIGURE 8

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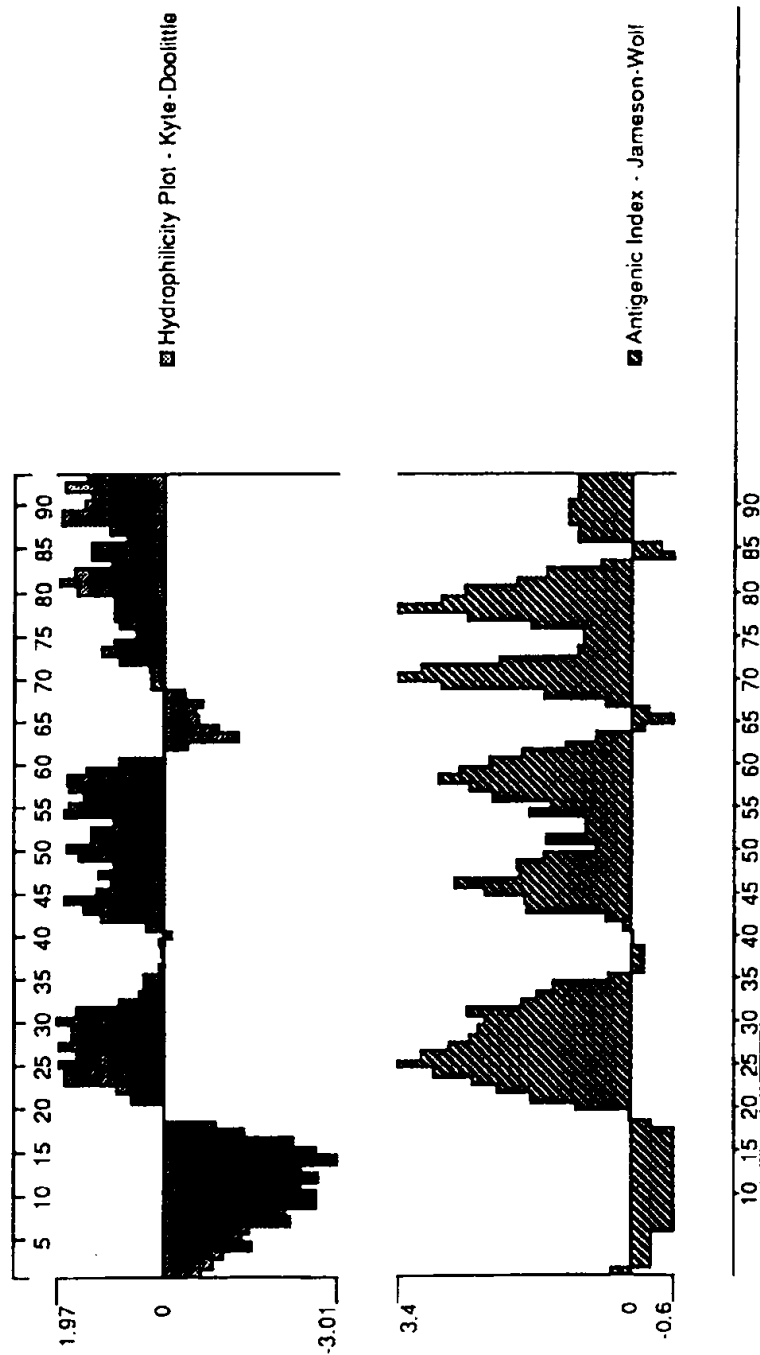


FIGURE 9



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$\Delta$  intracellular calcium in response to 111571  
THP-1

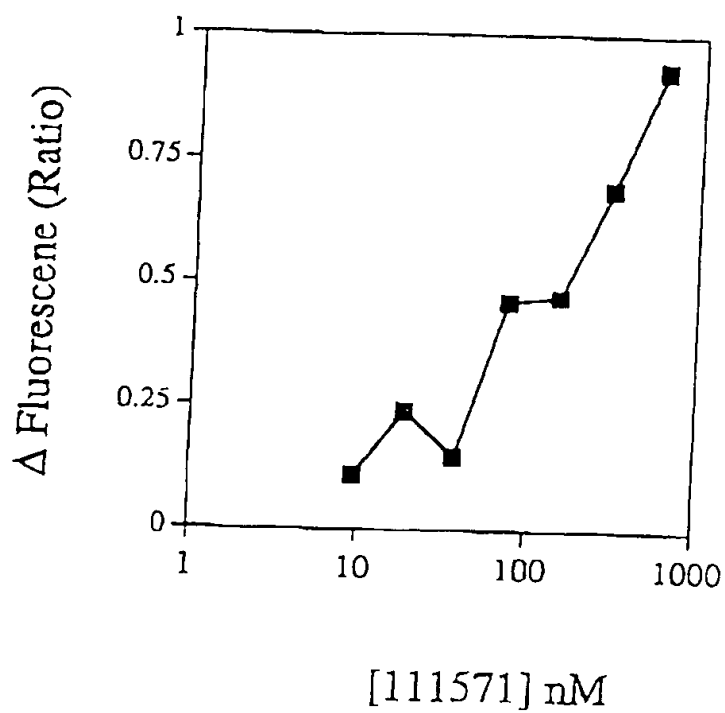


FIGURE 11

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Chemotaxis of THP-1 Cells in Response to 111571

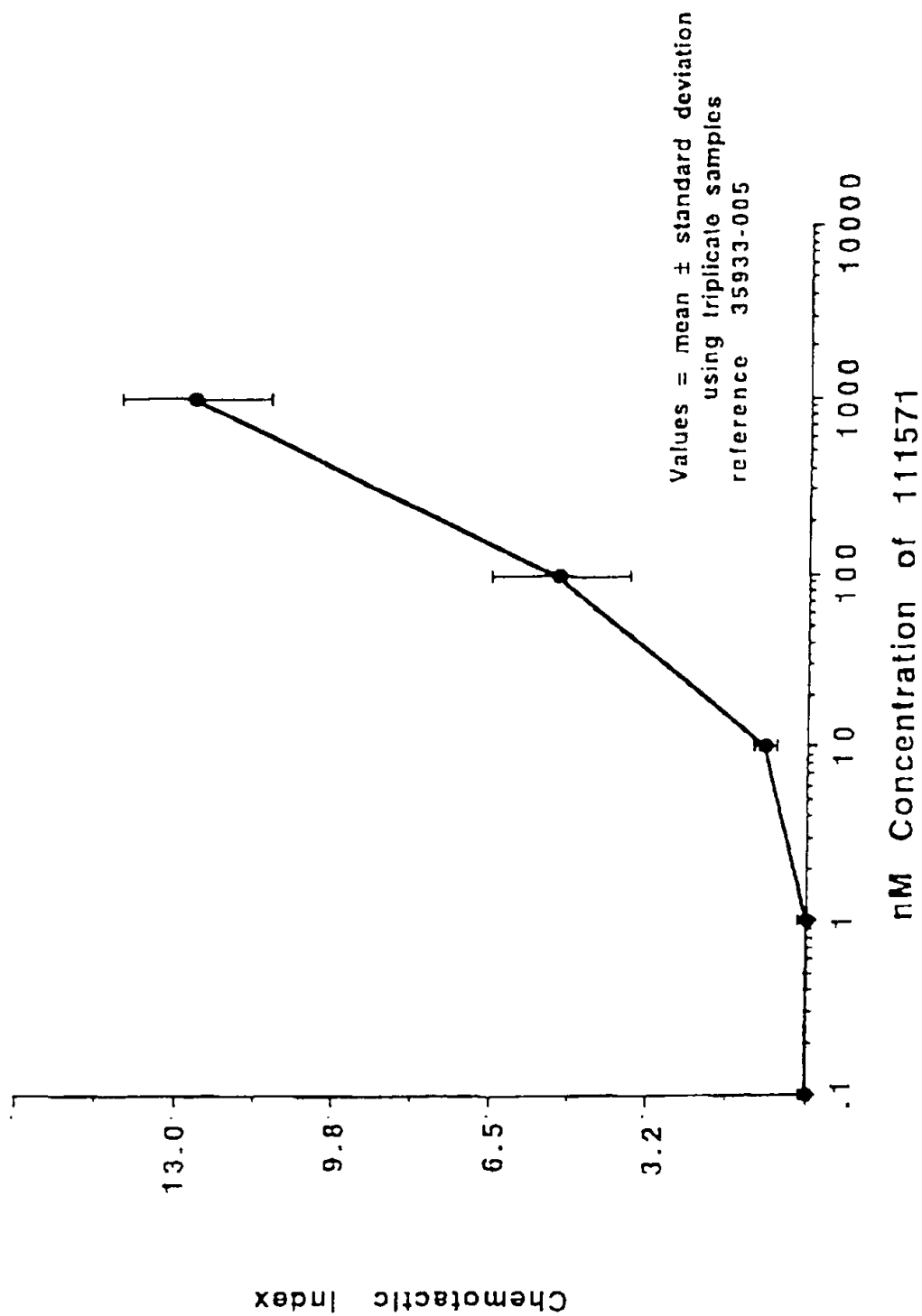


FIGURE 12

## CROSS-DESENSITIZATION OF 111571 + MIP-1 $\alpha$ in THP-1 CELLS

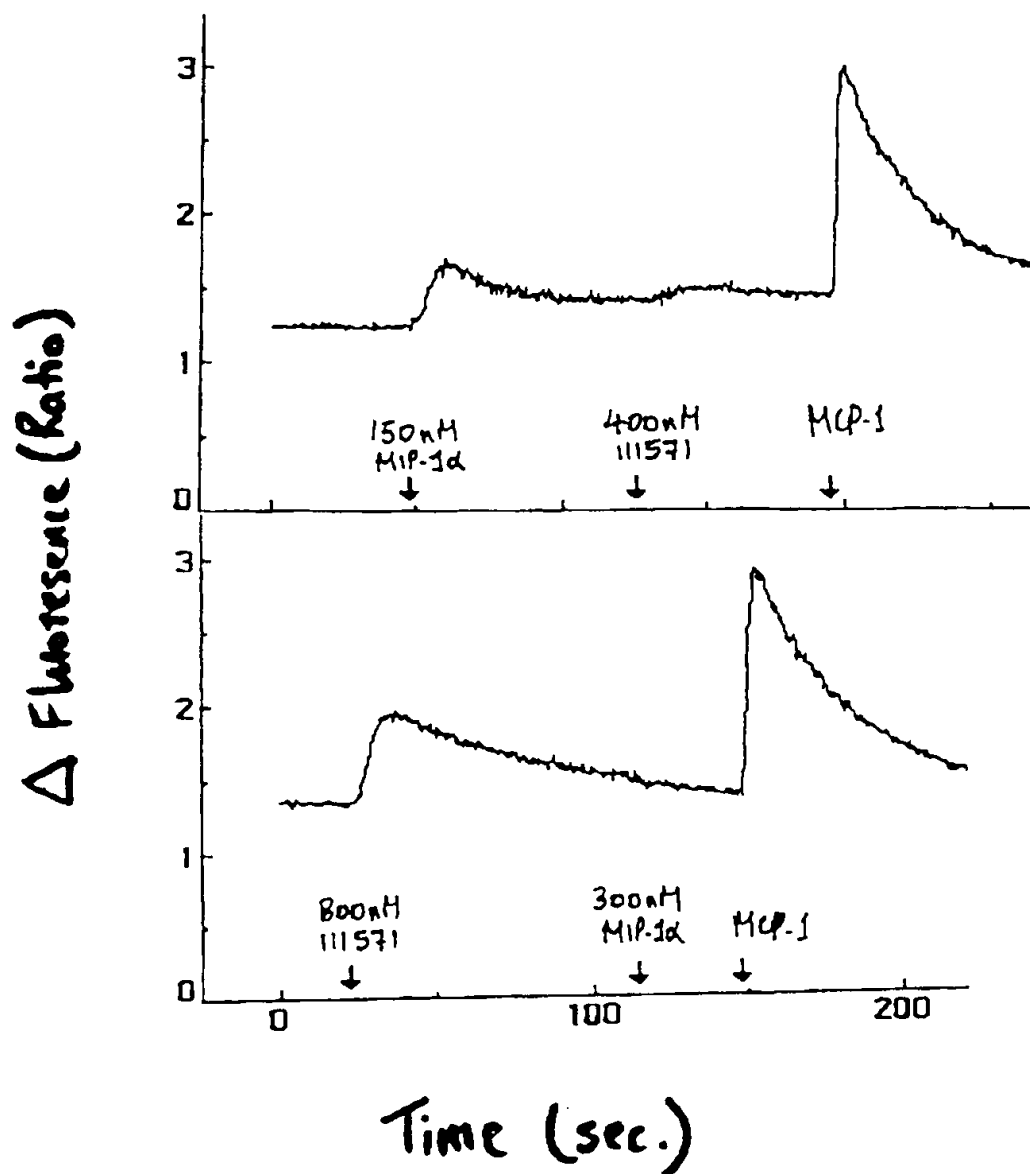


FIGURE 13

# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US95/15484

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : C07K 1/00, 16/00; C07H 21/04; C12Q 1/68; C12P 21/06; C12N 5/00  
US CL : 530/351, 387.1; 536/23.5, 24.31, 24.33, 24.5; 435/6, 69.1, 240.2  
According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 530/351, 387.1; 536/23.5, 24.31, 24.33, 24.5; 435/6, 69.1, 240.2

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

MPSRCH

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages                                                                                                                                            | Relevant to claim No. |
|-----------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------|
| A         | Proceedings of the National Academy of Sciences USA, Volume 85, issued December 1988, Lipes et al, "Identification, cloning, and characterization of an immune activation gene", pages 9704-9708, see the entire document.    | 1-22                  |
| X         | Gene, Volume 146, issued 1994, Matoba et al, "The addition of 5'-coding information to a 3'-directed cDNA library improves analysis of gene expression", pages 199-207, see Fig. 3 reference to GenBank accession no. D17181. | 3                     |
| ---       |                                                                                                                                                                                                                               | -----                 |
| A         |                                                                                                                                                                                                                               | 1, 2, 4-22            |

☐ Further documents are listed in the continuation of Box C.

☐ See patent family annex.

|                                                                                                                                                                        |    |                                                                                                                                                                                                                                              |
|------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| * Special categories of cited documents:                                                                                                                               | T  | later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention                                              |
| *A* document defining the general state of the art which is not considered to be part of particular relevance                                                          | X* | document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone                                                                     |
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| *P* document published prior to the international filing date but later than the priority date claimed                                                                 |    |                                                                                                                                                                                                                                              |

Date of the actual completion of the international search

14 MARCH 1996

Date of mailing of the international search report

25 MAR 1996

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